The role of non-ionic surfactant vesicles on the course of *Plasmodium chabaudi chabaudi* AS infection in BALB/c mice

by

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For my great mother, my sister, my brother and my dear husband

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Declaration

I declare that this thesis is of my composition and the work described herein was entirely done by me except where stated

Kowthar S. Hassan
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Abbreviations

B cell	B Lymphocyte
BSA	Bovine serum albumin
CD	Cluster of differentiation
Ci	Curi
CM	Complete Medium
ConA	Concanavalin A
cpm	Counts per Minute
CRP	C-Reactive Protein
CSP	Circumsporozoite Protein
DDT	Dichlorodiphenyltrichloroethane
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Foetal Calf Serum
HLA	Histocompatibility Leukocyte Antigen
ICAM-1	Intercellular adhesion Molecule-1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interluekin
i.p.	Intraperitoneally
i.v.	Intravenously
L-NMMA	L-NG-monomethyl arginine
LPS	Lipopolysaccharide
MHS	Major Histocompatibility Complex
mg	Milligram
µg	Microgram
MTT	(3-(4,5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide)
NISV	Non-ionic Surfactant Vesicles
NO	Nitric Oxide
nRBC	Non-infected red blood cells
PBS	Phosphate Buffer Saline
PBMN	Peripheral Blood Mononuclear Cells
pRBC	Parasitised Red Blood Cells
RPMI	Rosewell Park Memorial Institute
SE	Standard Errors
T cell	T Lymphocyte
TGFβ	Transforming Growth Factor Beta
Th	T Helper Cell
TNFα	Tumor Necrosis Factor alpha

Abstract

The pro-inflammatory cytokine, TNFα has been linked with several patologies like malaria, toxic shock and rheumatoid artheritis. Moreover the circulating levels of TNFα have been shown to correlate positively with disease severity in both murine and human malaria. Among clinical features thought to be produced by TNFα are fever and cachexia. TNFα, however, has also been shown to play a protective role in malaria. Indeed protection against the asexual stages of the murine malaria *Plasmodium chabaudi chabaudi* AS has been shown to depend on the sequential appearance of Th1 and Th2 cell functions with the Th1 stimulation leading to the early production of TNFα through activation of macrophages by IFNγ released from the Th1 cells. This indicates, therefore, that certain levels of this cytokine are essential for protection but excessive levels are detrimental to the host. Various methods have been used to suppress excessive levels of circulating TNFα levels in various pathologies. These include, the use of anti TNFα monoclonal antibodies and the use of soluble TNFα receptors.

In the studies presented here, a new method for suppressing circulating TNFα was investigated. It involves the use of empty non-ionic surfactant vesicles (NISV) which had been shown previously to cause a less degree of weight loss in *Toxoplasma gondii*-infected mice than infected but untreated mice. Since high circulating TNFα levels have been shown to be linked with cachexia, the mechanism suggested for the reduced weight loss in the *T. gondii*-infected mice was the possibility that empty NISV were suppressing the release of TNFα from the activated macrophages. In the present studies, empty NISV were used in *P. chabaudi chabaudi* AS-infected mice to investigate their potential in reducing the amount of weight loss caused by this particular infection. It was also investigated in these studies the hypothesis that the reduced weight loss was due to suppression of the TNFα production by NISV from the macrophages and the mechanisms involved in this suppression.

Experiments were repeated a number of times in 2 different animal houses (Glasgow University and Strathclyde University). Pre-infection treatment with NISV produced a

significantly less degree of weight loss ($p<0.05$) in the treated animals than their counterparts in the experiments conducted at Strathclyde University, while at Glasgow University, less weight loss was observed in the untreated group. Variability in a number of parameters (including diet, PABA content in water, coinfection of the animals with other pathogens) was considered in order to explain the discrepancy in the results but all parameters were identical in both venues. Furthermore, combining the results of the experiments conducted at each venue showed no significant difference between the 2 groups. Moreover, the time points where a significant difference in weight loss between the 2 groups was seen, were different in different experiments. The conclusion was that pre-infection treatment with empty NISV had no significant effect on the weights of *P. c. c* AS- infected BALB/c mice.

Levels of parasitaemia were also measured in both groups of all experiments. Discrepancy was also observed here between the experiments conducted at the 2 venues. Lower levels of parasitaemia were detected in the NISV-treated animals than the untreated ones at certain time points in individual experiments conducted at Strathclyde University. With the 2 experiments combined no difference was seen between the 2 groups. In the experiments conducted at Glasgow University, lower levels of parasitaemia were seen in the NISV-untreated group in one experiment and in the NISV-treated group in the second experiment on day 9 during the incline of parasitaemia. A higher parasitaemia was seen on day 11 in the NISV-treated group during the declining phase of parasitaemia. Again the time points where a difference was observed were different in different experiments indicating that the difference was not due to treatment with NISV. Also, taking the results together showed no significant difference between the 2 groups. The conclusion was that pre-infection treatment with NISV had no effects on the levels of parasitaemia in *P. c. c.* AS-infected mice.

A health status score was developed to monitor the animals closely. It consisted of degree of pilo erection, degree of activity of the animals, and level of self-hygiene. A significantly higher score was observed in the NISV-treated group in the experiments conducted at Strathclyde University on days 11 and 12 post infection. A significantly higher score on the other hand was seen in the untreated group in the experiments conducted at Glasgow University on days 12, 13 and 14. The conclusion was that

pre-infection treatment with NISV had no effect on the general health status of the *P. c. c* AS infected animals.

The effects of pre-infection treatment with NISV on plasma TNFα levels were studied in 3 of the above experiments. Results showed an apparent suppression in plasma TNFα levels in the NISV-treated group in all the 3 experiments. The significance of the suppression could not be confirmed due to absence of standard errors since samples of each group had to be pooled to allow multi-cytokine analysis. The observation that plasma TNFα levels were suppressed in the experiments yielding contradictory results with regards to weight loss, parasitaemia levels and health status ,indicated that either the NISV had no genuine suppressive effect on circulating TNFα levels or that NISV suppressed TNFα in an uncontrollable manner.

To investigate whether the above suppression was due to chance or a due to uncontrollable effect of NISV, NISV effects were further investigated in *in vitro* settings. NISV were found to induce production of TNFα from liver and bone marrow adherent cells. NISV were also found to induce the production of NO from splenocytes, and liver and bone marrow adherent cells in a dose dependent manner. NISV also caused suppression of splenocyte proliferation which partly was reversed by LNMMMA. Since the *in vitro* settings were different from the *ex-vivo* settings with regards to the time interval between NISV treatment and TNFα measurement, it was suggested to modify the experiments. NISV treatment should be administered at time of infection and plasma TNFα levels should be measured in the first 4 hours post infection to resemble the *in vitro* experiments. This suggested modification, however, was not done in these experiments due to shortage of time.

To investigate any possible immunoregulatory effects of NISV in the *P. c. c* AS-infected mice, plasma levels of cytokines representing activation of Th1 and Th2 arms of the immune response were measured. Plasma levels of IFNγ, IL-4 and IL-10 were measured in all experiments. No consistent difference was seen between the NISV-treated and untreated groups with regards to IFNγ or IL-4 levels. IL-10 levels were not reported due to problems with IL-10 standards. No consistent difference was

observed in the nitrate levels between the 2 groups. The conclusion was that pre-infection treatment with NISV had no effects on plasma IFNγ, IL-4 or nitrate levels.

The possibility of running ELISAs on whole lysed blood was also tested. It was found that it was indeed possible to detect TNFα, IFNγ and IL-4 in whole lysed blood samples by ELISA with a slight modification in the steps. After adding the samples to the wells, the plate was left at 4°C overnight instead of the usual 2 hrs.

To investigate the effect of haemolysis on the levels of cytokines detected by ELISA method, levels of TNFα and IFNγ were measured in samples of lysed whole blood, serum, plasma, lysed packed cells remaining after removal of plasma and lysed packed cells remaining after the removal of serum. Highest TNFα and IFNγ levels were detected in lysed packed cells remaining after removal of plasma.

Also investigated in these studies was the effect of repeated freezing and thawing on plasma samples. Different levels of the above cytokines were detected after repeated freezing and thawing. Higher IFNγ levels were detected after 6th time of thawing than after second thawing. The results indicate the importance of standardizing the method of measuring cytokines by ELISA and stating if repeated freezing and thawing had been involved.

The final collective conclusions of the studies presented here are that pre-infection treatment of *P chabaudi chabaudi* AS-infected BALB/c mice with empty NISV had no effect on the clinical features (weight, parasitaemia levels, general health status) of the treated animals. The treatment had no effects on the plasma levels of IFNγ, IL-4 and NO. *In vitro* settings, NISV induced the production of TNFα and NO and suppressed splenocyte proliferation to various stimulants. A modification of the *ex-vivo* experiments is required along with the use of standard errors to investigate whether the *ex-vivo* suppression of plasma TNFα was a chance finding or a genuine result of NISV. Finally, further studies are required to explore the use of whole lysed blood samples in cytokine measurements by ELISA.

Chapter 1

General Introduction

Background

Malaria remains one of the major threats to mankind today. It affects 300-500 million people and causes 1.5-2.7 million deaths every year half of which occur in children under five years of age in the sub-Saharan Africa (Trigg, 1998). Malaria also imposes a huge economic burden on the endemic areas, and seems to be linked with poverty in a vicious circle. Ill health caused by malaria impedes economic growth, which in turn prevents the implementation of measures required to combat the disease such as bednets, use of chemotherapy and insecticides (Sachs and Malaney, 2002).

The disease is not new. It was known to the ancients. Hippocrates, who lived in the fifth century BC, was the first to describe the clinical appearance of malaria and some of its complications. The disease was named malaria, which means bad air, by the Italians who believed it to be associated with the foul air near marshy areas (Gilles and Warrell, 1993). Malaria used to be widely distributed and malaria fever was reported in central Europe, much of Asia and North America. Its decline and eventual disappearance from the USA was due to improved health style with the use of screened homes, and programmes aiming at modifying the mosquito habitat. It also declined in central Europe but without any special efforts. In Southern Europe, Turkey and some parts of Asia it declined because of the use of (dichlorodiphenyltrichloroethane) DDT. In the 1950s and 1960s DDT was used in an attempt to eradicate the disease globally and indeed good results were obtained in several countries such as India, Sri Lanka and former USSR, while in sub-Saharan Africa no sustained effort was made to control the disease. In the early 1960s, however, and due to excessive use of DDT in agriculture, DDT-resistant mosquitoes appeared. At the same time, there was lack of funding in the eradication programme with the consequences of the disease re-emerging (Shiff, 2002). For the next 25 years, interest in malaria had fallen with only 3 of 1,223 new drugs between 1975-1996 were antimalarials, and support for malaria research declined. Furthermore, in many malaria-endemic areas, national malaria control programmes established during the colonial period collapsed (Greenwood and Mutabingwa, 2002). In recent years, the

malaria situation has deteriorated due to parasite resistance to anti-malarial drugs, vector resistance to insecticides and civil strife. Furthermore, modern easy travel, global warming and movement of refugees are causing a threat to areas considered previously as malaria-free (Greenwood and Mutingawa, 2002).

The Parasite

Malaria is caused by a protozoan which is classified as follows: phylum: Apicomplexa, class: Sporozoasida, subclass: Coccidiasina, order: Cossidiasina, suborder: Haemospororina, genus: *Plasmodium* (Murray *et al.*, 1990). It was first identified and described by Laveran who in 1880 described malaria parasites in human red blood cells (Gilles and Warrell, 1993). There are about 120 species of *Plasmodium* (Gilles and Warrell, 1993) but only four of them routinely infect humans. This is because the parasite is generally host specific, i.e. certain plasmodial species cause disease in certain host species. It must be mentioned, however, that *P. falciparum* which is a human infecting plasmodial species, can infect some South American monkeys such as *Aotus* and *Saimiri* spp and some monkey malaria parasites such as *P. cynomolgi*, and *P. knowlesi* can infect humans (Garnham, 1967).

The four species that infect humans are *P. falciparum, P. vivax, P. malariae* and *P. ovale*. *P. falciparum* and *P. vivax* contribute most heavily to the malaria morbidity, while most of the deaths are attributed to *P. falciparum* which is, therefore, considered clinically as the most important species. A diagrammatic summary of the parasite life cycle is illustrated in figure 1.1.

Malaria is transmitted by female anopheline mosquitoes. There are about 400 species of *Anopheles*, 60 of which have been proven as vectors for human malaria (Gilles and Warrell, 1993). The involvement of mosquitoes in malaria transmission was identified by Ronald Ross in 1897. Ross found a developing form of malaria parasites in the body of a mosquito that had fed on the blood of a malaria patient (Gilles and Warrell, 1993). It is

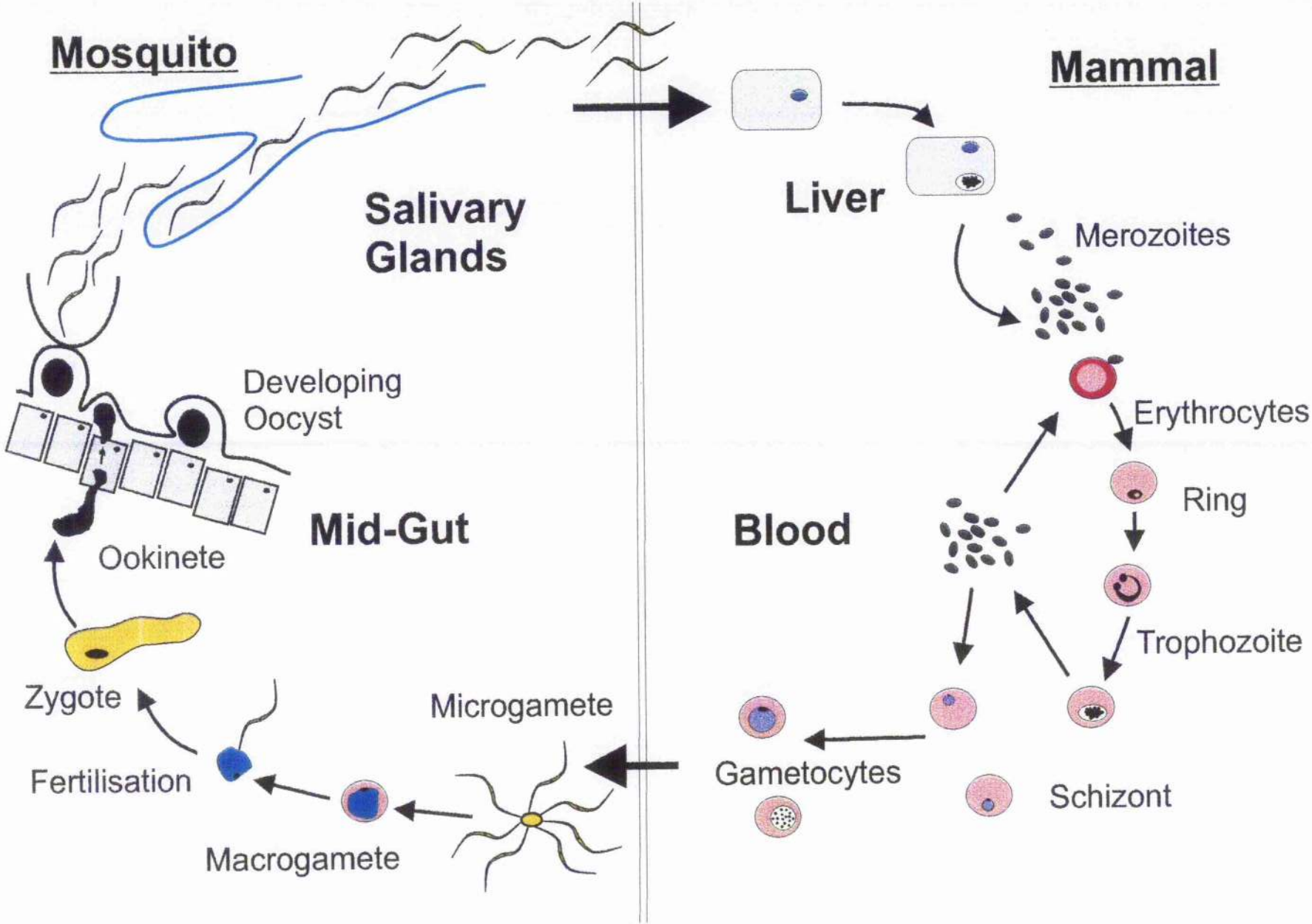


Diagram 1.1 The life cycle of *Plasmodium* species

said that mosquito involvement was suggested to Ross by Manson who had described the mosquito as the arthropod host for human filarial in 1878 (Sherman 1998). It must be mentioned however, that Al-Mutanabbi (915-965 AD) an Arab poet, while in Egypt described himself suffering from a well known common disease characterized by rigors and caused by mosquitoes biting at night. This disease, was said to be malaria (school teacher, personal communication) (see Appendix 3 for part of the poem).

Mosquito's role in the spread of the disease depends on several factors including the temperature and humidity of the environment, which determine the longetivity of the mosquitoes, and also the rate of parasite development inside the vector and hence the completion of the parasite life cycle. The period required for completion of parasite life cycle increases as the ambient temperature declines, with development ceasing completely below 16^{0}C (Gilles and Warrell, 1993).

The cycle starts when the female *Anopheles* mosquito introduces its proboscis into a blood vessel to feed on the host's blood. This is important for the development of her eggs while the male feeds on nectar and other plant juices (Gilles and Warrell, 1993). During this process she injects her saliva containing an anticoagulant into the host to facilitate blood uptake. Unfortunately for the host, if any sporozoites exist in the saliva, they are also injected into the circulation. Recently it was shown that *P. berghei* in mice infection occurred when sporozoites were injected into skin, muscle, peritoneal cavity and tail end of mice. Animals also developed the infection when *A. stephensi* was forced not to take blood but only to probe into the skin. Sporozoites that were injected into skin were found to remain at the site of injection for at least 5 minutes and later appear in the circulation (Matsuoka *et al.*, 2002). The released sporozoites are cleared from the circulation within an hour as Fairlay's work showed in 1946 (reviewed by Meis and Verhave, 1988) and gain access into the hepatocytes where they develop (Shortt and Garnham, 1948). Subsequent work done to examine the interaction of sporozoites and Kupffer cells by transmission electron microscopy showed that in fact 10-15 minutes following inoculation sporozoites are seen both free in sinusoids and inside Kupffer cells (Meis *et*

al., 1983). Recent work has shown that sporozoites pass through several hepatocytes before intrahepatic parasite development is established (Mota *et al.*, 2001). Carrolo *et al* have reported on the significance of the sporozoite journey throught several hepatocytes. They have shown that while the sporozoites travel through the heaptocytes they wound these hepatocytes causing the release of hepatocyte growth factor (HGF). HGF then binds to its receptor and the complex mediates rearrangement of the hepatocyte actin cytoskeleton rendering the cells susceptible to infection (Carrolo *et al.*, 2003). The authors suggested important clinical implications for their findings in developing new strategies against the disease by inhibiting HGF synthesis or its activation. Their findings, however, may be difficult to reconcile with findings reported previously in the same year by Kaiser *et al.* This group has found that *P. berghei* sporozoites that reside in the salivary glands of mosquitoes can transform into the exoerythrocytic forms without residence in the host hepatocytes. Their transformation was dependent on serum and temperature of 37°C and did not require hepatocytes or specific host cell-derived growth factors (Kaiser *et al.*, 2003). Most of the bites by infected mosquitoes results in inoculation with only a few sporozoites although the salivary glands frequently contain several thousand sporozoites (Beier *et al.*, 1991).

Sporozoites are found to exhibit gliding motility associated with the secretion of the circumsporozoite protein (CSP) from their apical end and the translocation of CSP posteriorly (Stewart and Vanderberg, 1991). The mechanisms through which the parasites manage to gain access into hepatocytes are unknown. Since the hepatic sinusoidal endothelium has small fenestra of 0.1μm in diameter (Wisse, 1985) and since the sporozoites are 1x15μm in size, it was thought that access of sporozoites through sinusoidal fenestra was not possible. Studies on *P. berghei*-infected rats showed a role for Kupffer cells in delivering the sporozoites from the sinusoids to the space of Disse and the bordering hepatocytes. Impairment of Kupffer cells function by silica treatment led to significant prolongation of circulating time of sporozoites and a significant reduction in the number of developing schizonts (Verhave *et al.*, 1980). In 1983 Meis and colleagues examined the interaction between *P. berghei* sporozoites and rat Kupffer cells using

transmission electron microscope. They found that sporozoites were found inside endocytotic vacuoles of Kupffer cells and that their morphology was intact even one hour later with no signs of lysosomal digestion. They, therefore, suggested that Kupffer cells played an important role in delivering the sporozoites to the space of Disse and that endothelial cells had no role. How exactly do sporozoites enter the hepatocytes is still not known but it has been shown that the sporozoites bind through the region II of their CSP to heparan sulphate proteoglycans (HSPGs) on the hepatocyte's surface (Nussenzweig *et al.*, 1985). This binding on its own, however, is not enough and a mechanism for internalization is needed. The natural ligands of the HSPGs are chylomicrons and very low density lipoproteins (VLDL) which, following binding to HSPGs, are internalized by the low density lipoprotein (LDL) receptor and the LDL receptor related protein (LRP). It was shown that CSP also binds to LRP, therefore raising the possibility that sporozoites may depend on coordinated activity of HSGP and LRP (Shakibaei *et al.*, 1996). Subsequent studies, however, have shown that sporozoites of *P. yoelii yoelii* infect LRP-deficient hepatocytes *in vivo* and LRP-null cells *in vitro* with efficiency equal to controls indicating that LRP is not required by *Plasmodium* sporozoites to enter the hepatocytes (Marshall *et al.*, 2000).

Thrombospondin related adhesion protein (TRAP) which is found in the micronemes as well as on the surface of salivary gland sporozoites (Rogers *et al.*, 1992,) has also been shown to be involved in sporozoite motility and hepatocyte invasion (Rogers *et al.*, 1992, Gantt *et al.*, 2000). Sporozoites of *P. berghei* with a disrupted TRAP gene were not capable of gliding motility or hepatocyte invasion (Kappe *et al.*, 1999, Sultan *et al.*, 1997). Recently, *Plasmodium falciparum* erythrocyte membrane protein 3 (PfEMP3) knob-associated protein was found on the surface of sporozoites and in the cytoplasm of the liver stages (Grüner *et al.*, 2001). Specific antibodies obtained from individuals living in malaria endemic areas blocked invasion of *P. yoelii yoelii* and *P. berghei* sporozoites into hepatocytes *in vitro* (Grüner *et al.*, 2001).

Within the hepatocytes, sporozoites develop and replicate forming the liver schizont, which ruptures releasing merozoites into the circulation. Depending on the *Plasmodium* species, 100-30,000 merozoites are released from the initial hepatoschizogony (reviewed by Meis and Verhave, 1988). The pre-erythrocytic cycle varies in duration among the different species taking 5.5-6 days for *P. falciparum*, 10 days for *P. vivax* and 2 weeks for *P. malariae* (reviewed by Meis and Verhave, 1988).

Sporozoites of some species including *P. vivax and P. ovale* can lie dormant within the hepatocytes and hence called hypnozoites (Krotoski, 1985). Hypnozoites persist in the hepatocytes as a small uninucleate form 4-5mm in diameter until reactivation with a subsequent relapse of the disease (reviewed by Meis and Verhave, 1988). What causes the reactivation of these hypnozoites is still unknown.

Invasion of red blood cells:

The merozoites released from the hepatocytes invade red blood cells. Different species may have different preferences with respect to the age of red blood cell invaded. For instance *P. vivax* invades reticulocytes, *P. falciparum* can infect erythrocytes of any age but prefers young ones (Pasvol *et al.*, 1980) and *P. malariae* infects mature erythrocytes. Early work on *P. yoelii* and *P. gallinaceum* showed that the process of invasion involves several steps beginning with the parasite initially making contact with the host red blood cell membrane and reorientating itself so that its anterior end comes in contact with the erythrocyte. At this site of contact a depression is formed in the red cell membrane, which deepens as the merozoite moves inwards. Eventually the parasite is internalized within a vacuole inside the erythrocyte called the parasitophrous vacuole (Ladda *et al.*, 1969). The entire process of erythrocyte invasion takes less than 30 seconds (Pasvol *et al.*, 1995). From the above description, it looks that the process of invasion is not that of a penetration but rather of invagination resembling the implantation of a human zygote into the uterine wall.

It was previously thought that the initial contact between the parasite and the red blood cell did not require specific red cell receptors since treatment of red blood cells with trypsin, chymotrypsin and neuraminidases did not affect the ability of the parasite to invade these cells (Sherman, 1966). Now we know this is not true and indeed the presence of specific molecules on the red blood cells are required to allow invasion to occur. For example, in the case of *P. vivax*, Duffy blood group antigens are required for the process of invasion and this explains why those Africans negative for Duffy antigens are resistant to *P. vivax* infection (Miller *et al.*, 1976). In Duffy negative individuals, the initial attachment of the merozoites to the erythrocyte membrane does occur, but the merozoites are unable to form a junction with the erythrocyte membrane (Spencer *et al.*, 1978). *P. falciparum*, on the other hand, requires the presence of glycophorins which are carbohydrate rich glycoproteins traversing the erythrocyte membrane, since monoclonal antibodies against an epitope on glycophorin A markedly inhibited red cell invasion by merozoites *in vitro* (Pasvol *et al.*, 1982, Thompson *et l.*, 2001). Sialic acid residues of glycophorin A have been identified as invasion receptors for *P. falciparum* (Miller *et al.*, 1977, Pasvol *et al.*, 1982). The parasite ligand for sialic acid residues is the erythrocyte binding antigen-175 kDa (EBA-175) (Camus and Hadley*e*, 1985). *In vitro* studies, however, have shown that some clones of *P. falciparum* can invade erythrocytes using sialic acid-independent pathways (Dolan *et al.*, 1994). *P. falciparum* field isolates were also commonly found to use erythrocyte invasion pathways that are independent of sialic acid residues of glycophorin A (Okoyeh *et al.*, 1999).

The merozoite membrane proteins involved in erythrocyte invasion include, the merozoite surface protein-1 (MSP-1) and ring stage surface antigen (RESA) which interact with spectrin (Herrera *et al.*, 1993, Foley *et al.*, 1991), apical membrane antigen-1 (AMA-1) (Triglia *et al.*, 2001), erythrocyte binding antigen (EBA-175) which binds to glycophorin A (Sim *et al.*, 1994) and acidic-basic repeat antigen (ABRA) which interacts with Band 3 (Kushwaha *et al.*, 2002). Microtubules of *P. falciparum* have also been shown to take part in the invasion of merozoites into erythrocytes since colchicin which, inhibits polymerization of microtubules inhibited erythrocyte invasion by *P. falciparum*

in vitro (Bejon *et al.*, 1997). Other proteins identified on the surface of *P. falciparum*-infected erythrocytes that are involved in the process of invasion include *P. falciparum* rhoptry protein homologous PfRH2a and PfRH2b (Rayner *et al.*, 2000, Triglia *et al.*, 2001). Parasite proteases are also thought to play a part in erythrocyte invasion and erythrocyte rupture during schizogony (Rosenthal *et al.*, 1998). Proteases from *P. falciparum*, *P. chabaudi* and *P. berghei* have been identified to interact with and cleave erythrocyte proteins such as Band 3, glycophorin, ankyrin, protein 4.1 and spectrin (Raphael *et al.*, 2000, Braun-Breton *et al.*, 1992, Roggwiller *et al.*, 1996)

Following invasion of red blood cells

Following invasion of the red blood cells each *Plasmodium* species induces characteristic morphological changes, both within and on the surface of infected cells, related to the development of the parasite. Some of these changes include the appearance of membrane bound clefts called Maurer's clefts in the cytoplasm of the erythrocytes infected with *P. falciparum* (Aikawa, 1988). Maurer's clefts connect to the membrane of the parasitophorous vacuole and appear to increase in number as the parasite matures (Mackenstedt *et al.*, 1989). Another change is the formation of knobs by the asexual erythrocytic stages of *P. falciparum*. These knobs start appearing approximately 24hr following merozoite invasion and may cover as much as 5% of the surface of the infected cell (Gruenberg, Allerd, and Sherman, 1983). Under scanning electron microscopy, the knobs are seen to vary in size and density, being sparse on trophozoite containing cells and more numerous and of smaller size on schizont-infected cells (reviewed by Sherman, Crandall and Smith, 1992). The role of the knobs is not really known for they were initially thought to be essential in the phenomenon of cytoadherence and electron microscopy clearly showed *P. falciparum*-infected red cell cytoadhering to the endothelium via their knobs (Aikawa, 1988). It has also been shown, however, that *P. malariae* which does not cytoadhere, does form knobs and *vice versa* in the case of the rodent parasite *P. chabaudi* (Cox, Semoff and Hommel, 1987).

Once inside the red blood cells the parasites can take one of two directions, asexual proliferation or differentiation into sexual forms (Killick-Kendrick and Warren, 1968). In the process of asexual proliferation, the young ring stage parasites develop into trophozoites and then into the dividing schizont, which ruptures giving 6-32 merozoites depending on the *Plasmodium* species. The released merozoites invade more erythrocytes and the whole cycle is repeated. The escape of merozoites from the erythrocytes has been shown to occur in two steps. The first step involves the release of merozoite bound by the parasitophorous vacuolar membrane (PVM) from the erythrocytes and then the escape of the merozoites from the PVM (Salmon *et al.* 2001). Both these steps are protease dependent, since treatment of *P. knowlesi*-infected erythrocytes with chymostatin allowed the development of the parasites but not their release from the erythrocytes (Hadely *et al.*, 1983). Also, culture of *P. falciparum* schizonts-infected erythrocytes in the presence of the cysteine protease inhibitor, L-transepoxy-succinyl-leucylamido-(4-guanidino) butane (E64) resulted in the accumulation of extraerythrocytic merozoites locked within a thin, transparent membrane which was shown to be derived from the PVM (Salmon *et al.*, 2001). The asexual cycle varies in duration among the species, for example being 24 hr long in the case *P. chabaudi* and 48 hr in *P. falciparum* (reviewed by Meis and Verhave, 1988). The other option for the parasites is to develop into microgametocyte males or macrogametocyte females. The gametocytes are taken up by the feeding mosquito along with other forms during a blood meal. The signals that determine whether a merozoite will develop asexually or sexually *in vivo* are not known although several stimuli were proposed such as increase in asexual parasitaemia *per se*, rising immunity to asexual stags, a fall in immunity against sexual stages, a change of spleen pH, presence of parasite cell debris and sub-curative levels of anti-malarial drugs like quinine (reviewed by Sinden, 1983). *In vitro* work, on the other hand, has shown that, dilution of ring stage-infected erythrocyte cultures with fresh erythrocytes suppressed gametocytogenesis (Carter and Miller *et al.*, 1979), while addition of cAMP may increase it (Kaushal *et al.*, 1980). There is some evidence, however, that in *P. falciparum* a given erythrocytic schizont contains either all sexual or asexual merozoites indicating that the trigger for differentiation precedes invasion. The rate at which the merozoites differentiate into

gametocytes varies considerably during successive rounds of schizogony. In *P. falciparum*, gametocytogenesis takes about 10-12 days which is much longer than the 2 days of the asexual cycle (reviewed by Sinden, 1983). Most of the lifetime of gametocytes in *P. falciparum*, the immature gametocytes are sequestered, possibly in the bone marrow (Smalley *et al.*, 1981).

As the mosquito sucks blood of the host, it takes in various forms of the parasite. Once inside the midgut of the mosquito, the asexual forms are digested together with the red blood cells, while the sexual ones undergo the process of gametogenesis. This is triggered by a drop in temperature below 30°C with subsequent rise in pH and unidentified mosquito midgut factors (reviewed by Sinden, 1983). With respect to mosquito midgut factors, a factor referred to as gametocyte-activating factor (GAF) was found in the midgut of female mosquitoes and is thought to be the natural inducer of *Plasmodium* gametogenesis (Garcia *et al.*, 1997, Billker *et al.*, 1997). Fusion of the two sexual forms in fertilization leads to the formation of the diploid zygote, which is the only diploid form of the parasite throughout its entire life cycle. The zygote, which is a non-motile round body, undergoes a rapid meiosis leading to the generation of the haploid mobile, elongated ookinetes (Gilles and Warrell, 1993) These newly developed ookinetes have to escape the hostile environment of the blood meal which contains antibodies, leukocytes and complement (Lensen *et al.*, 1997, Tirawanchai *et al.*, 1991). Ookinetes penetrate the midgut wall into the haemocele (Sinden *et al.*, 1996). *In vitro* culture systems showed ookinetes of *P. berghei* to invade the midgut cells at their lateral apical surface followed by invasion of neighbouring cells with the invaded cell dying rapidly by apoptosis (Zieler and Dvorak, 2000). Circmsporozoite-and TRAP-related protein (CTRP) an ookinetes microneme protein (Yuda *et al.*, 1999) was shown to be essential for this invasion. Disruption of its gene in *P. berghei* reduced ookinte gliding motility on glass surface, and inhibited invasion of mid gut wall as seen by absence of oocyts (Dessen *et al.*, 1999). On the outer side of the midgut wall, ookinetes mature into oocysts which begin to develop in the vector 24-72hr post ingestion of infected blood meal (Meis and Verhave, 1988). Depending on the parasite species and the temperature, thousands of sporozoites are

released from the mature oocyts into the haemocoele within 7-15 days of blood meal (Awono-Ambene *et al.*, 1998). Sporozoites travel to the salivary glands of the mosquito. When the mosquito feeds on blood, the sporozoites are injected into the host circulation and the whole life cycle is repeated.

During the parasite life cycle within the host, three phenomena have been reported with significance in the disease severity. These are, antigenic variation, cytoadherence and rosetting, which are mentioned here because of their importance.

Antigenic variation

Antigenic variation refers to a process of controlled phenotypic variation of surface-exposed antigenic determinants without introducing changes in the genome. It is a prime immune evasion mechanism employed by a number of pathogens such as *Plasmodium* and *Trypanosoma* to maintain a chronic infection (Borst and Greaves, 1987, Miller *et al.*, 1994). In *P. falciparum*, protein PfEMP1 which is expressed on the surface of infected erythrocytes and which mediates adhesion to other erythrocytes and to endothelial cells, is clonally variant (Baruch *et al.*, 1995, Rowe *et al.*, 1997, Chen *et al.*, 1998). This protein is encoded by a set of *var* genes which are distributed through out all the 14 chromosomes of the haploid *Plasmodium* genome and each parasite contains approximately 50-150 *var* genes (Su *et al.*, 1995, Rubio *et al.*, 1996, Fischer *et al.*, 1997, Hernandez-Rivas, 1997). Infected erythrocytes express only a limited number of *var* gene variants at any given time and *var* gene switching occurs at a rate of approximately 2% per generation *in vitro* (Roberts *et al.*, 1992, Smith *et al.*, 1995, Rowe *et al.*, 1997, Chen *et al.*, 1998). Two additional variant proteins are expressed on the surface of infected erythrocytes and undergo clonal variation. They are encoded by the *rif* and *STEVOR* genes (Kyes *et al.*, 1999, Fernandez *et al.*, 1999). A technique established for examining *var* gene expression has shown that ubiquitous transcription of all *var* gene variants occur in early ring stages. In trophozoite stages, *var* gene expression is tightly regulated by a silencing mechanism (Scherf *et al.*, 1998). The clinical importance of antigenic variation is that it imposes a

great challenge on the host's body which constantly has to recognize new antigens and therefore start an immune reaction all over again.

Cytoadherence

Cytoadherence is the process whereby red blood cells infected with mature parasites bind to endothelial cells in post-capillary venules (Udeinya *et al.*, 1981). Erythrocytes infected with mature asexual stages (trophozoites and schizonts) of *P. falciparum* bind to endothelial cells of post-capillary venules of many organs such as the brain, heart, lungs, small intestine and in the placenta in humans (Aikawa *et al.*, 1990, Pongponratn *et al.*, 1991). In the *Aotus* monkeys they are found primarily in the heart, adipose tissue, skeletal muscle and submucosa of the small intestine (Miller, 1969). This phenomenon is responsible for the presence of ring and non-pigmented young stages only in the peripheral blood as reported by Bignami and Bastinelli in 1890 (reviewed by Crandall and Sherman, 1994).

Cytoadherence is thought to help the mature erythrocytic forms avoid splenic filtration (Langreth and Peterson, 1985, Barnwell, 1989). It must be emphasized, however, that those parasites that do not exhibit cytoadherence like *P. malariae* also manage to undergo successful proliferation. Another suggested function for cytoadherence is to provide relatively low oxygen tension and high carbon dioxide tension found in post-capillary venules which are beneficial for the development of *P. falciparum* in *in vitro* culture (Scheibel *et al.*, 1979).

Cytoadherence is thought to play a vital part in the pathogenesis of severe malaria including cerebral malaria (Berendt *et al.*, 1994) through causing mechanical obstruction to blood flow and subsequent hypoxia (Aikawa *et al.*, 1990). Another suggested mechanism for damage caused by cytoadherence is the localisation of any released parasite toxins or indeed proinflammatory damaging cytokines in a particular area (Pasvol *et al.*, 1995). Furthermore, local release of nitric oxide by cerebral endothelial cells is

thought to interfere with the normal neurotransmission leading to coma (Clark *et al.*, 1992).

In the case of *P. falciparum* several endothelial proteins have been identified as receptors for the infected red cells, among these are intracellular adhesion molecule-1 (ICAM-1) (Berendt *et al.*, 1989), vascular adhesion molecule-1 (VCAM) (Ockenhouse *et al.*, 1992), CD36 (Barnwell *et al.*, 1989, Oquendo *et al.*, 1989), thrombospondin (Rock *et al.*, 1988), E-selectin (Ockenhouse *et al.*, 1992), the syncytiotrophoblast chondroitin sulphate (Rogerson *et al.*, 1995) and hyaluronic acid (Beeson *et al.*, 2000). The parasite-infected red cell surface molecule which mediates this binding is the variable *Plasmodium* falciparum erythrocyte membrane protein 1(PfEMP1) (Howard *et al.*, 1988, Leech *et al.*, 1984, Reeder *et al.*, 1999) which can bind to any of the above receptors simultaneously through its multiple adhesion domains. Variability in the disease severity among infected individuals has been attributed to variation in both the parasite adherence capacity to the host endothelium and to the ICAM-1 polymorphism in the host. For instance, a certain mutation in ICAM-1 has been shown to be associated with a greater risk of cerebral malaria in Kenyan children although no such association was found in Gambian children (Fernandez-Reyes *et al.*, 1997, Bellamy *et al.*, 1998). This is thought to be due to the *P. falciparum* isolates from Kenya having a greater binding tendency to ICAM-1 than the isolates from Gambia (Adams *et al.*, 2000).

Cytoadherence also occurs in the immature developing sexual stages of the parasite but they preferentially adhere to the vasculature of the spleen and the bone marrow (Smalley *et al.*, 1976). These forms start to adhere within 24hr following red cell invasion and remain there for 8-10 days until reaching stage V of their development when they re-enter the circulation and are capable of transmission through mosquitoes (Berendt *et al.*, 1990). Cytoadherence of the sexual forms is thought to be important for their development into the mature stages. Among the receptors used by the sexual forms to bind to the endothelium are ICAM-1 and CD36 (Rogers *at al.*, 1996) while Band 3 has been

suggested to be the ligand on the infected red cells responsible for cytoadherence (Crandall *et al.*, 1993, Rogers *et al.*, 1996).

Rosetting:

Rosetting describes a phenomenon in which uninfected erythrocytes adhere to trophozoite and schizont-infected erythrocytes in *in vitro* conditions. Up to 10 uninfected erythrocytes can be seen attached to a single infected erythrocyte and giant rosettes made of 20-30 cells have occasionally been reported. Rosetting was originally noticed in the simian malaria parasite *P. fragile* (David *et al.*, 1988) and later seen in several *Plasmodium* species including *P. falciparum* (Handunnetti *et al.*, 1989, Udomsangpetch, 1989), *P. coatnely* (Udomsangpetch *et al.*, 1991), *P. vivax* (Carlson *et al.*, 1990) and *P. chabaudi* (Udomsangpetch *et al.*, 1995). The molecules involved in rosetting include, CD36 and PfEMP1 (Handunnetti *et al.*, 1992).

The clinical significance of rosetting was investigated by several groups and the results were not always in agreement with each other. In the case of cerebral malaria in children from Papua New Guinea, no correlation was found between the degree of rosette formation and disease severity (Al-Yaman *et al.*, 1995) while a positive correlation was noted in children with cerebral malaria in Gambia (Carlson *et al.*, 1990) and in patients from Malagasy (Ringwald *et al.*, 1993). In the case of malaria in pregnancy, it was found that rosette forming-isolates are rarely found in the placenta indicating that rosetting did not correlate with disease severity here (Rogerson *et al.*, 2000).

It must be emphasized, however, that rosette formation is an *in vitro* phenomenon and its occurrence *in vivo* has not been reported yet. Hence results obtained from studies of rosetting must be interpreted with caution.

Recently another phenomenon was described and thought to be involved in determining disease severity. This is the agglutination of infected erythrocytes through platelets (Pain *et al.*, 2001). This platelet-dependent clumping of infected erythrocytes was found to occur through the platelet surface protein CD36. It was also found to be common in field isolates of *P. falciparum* from Kenya and to be associated with severe malaria (Pain *et al.*, 2001)

Epidemiology:

Occurrence of a disease is described by two main terms, epidemic and endemic. Epidemic malaria describes a sharp rise of the incidence of malaria among a population in which the disease was not known or an increase in clinical malaria in an area moderately endemic, due to some introduced factor. Endemic malaria is used when there is a consistent measurable incidence both of cases of the disease and of its natural transmission in an area over a succession of years. It is classified in the following way:
Holoendemic representing constant high exposure (perennial transmission of high degree resulting in a considerable degree of immune response in a large proportion of adults), hyperendemic describing high but seasonal exposure (immunity here is insufficient to prevent the effects of malaria on all age groups),
mesoendemic is found typically among small rural communities in the subtropical zones with varying intensity of transmission depending on local circumstances and hypoendemic where there is little transmission and the effects of malaria on the general population are unimportant (Gilles and Warrell,1993).

Clinical Malaria:

Malaria can present in a wide spectrum of clinical features ranging from asymptomatic parasitaemic individuals to death due to multiorgan failure. Among clinical conditions caused by malaria are:

Anaemia

Severe malaria is one of the complications of falciparum malaria. WHO has defined severe malaria as a Hb concentration of < 50g/L or a haematocrit < 0.15 in the presence of a *Plasmodium falciparum* parasitaemia > 10000 parasites /μl in a normocytic blood film. (Warrell, D. A. *et al.*, 1990).

The anaemia of malaria is multifactorial, involving both increased destruction and decreased production of erythrocytes. Destruction of the infected erythrocytes occurs at the time of schizont rupture (Greenwood *et al.*, 1991) and also there is premature removal of non-infected red cells by the spleen (Maggio-Price *et al.*, 1985). In addition, there is bone marrow suppression although the haematopoetic growth factors such as IL-3 and erythropoietin are normal or even elevated (Villeval *et al.*, 1990).

The relative contribution of the above mechanisms in the development of anaemia in malaria depends on the age of host, pregnancy status, anti-malarial immune status, genetic background of the host and the endemicity of the disease. In general it is thought that haemolysis plays the bigger part in non-immune children with acute malaria, while dyserythropoeisis is seen in individuals experiencing recurrent or frequent falciparum malaria although in any one individual several mechanisms are likely to operate (reviewed by Medez, Fleming, and Alonso 2000).

Cerebral malaria

Cerebral involvement in the form of convulsions or impaired consciousness in falciparum malaria can arise due to several factors. These are; side effects of chemotherapy, electrolyte imbalance due to vomiting, fever and hypoglycaemia (due to severe malaria per se or quinine therapy). All the above factors can impair consciousness and/or induce epileptic fits.

The term cerebral malaria (CM), however, is restricted to unarousable coma in *P. falciparum* positive patients and after excluding other causes of encephalopathy. Cerebral malaria occurs in approximately 1% of patients infected with *P. falciparum* and carries a mortality of approximately 15 % in children and 20% in adults (Warrell *et al.*, 1997, Allen *et al.*, 1996, Hien *et al.*, 1996).

At one point CM was thought to be due to changes in the blood brain barrier (BBB) permeability leading to intracerebral oedema (Maegraith and Fletcher 1972). Based on this hypothesis, patients with cerebral malaria were given a dexamethasone infusion but contrary to the expectations then, there was no difference in mortality between the treated and the untreated groups while coma was prolonged in the treated group. This led to abandonment of ancillary corticosteroid therapy and rejection of the permeability hypothesis (reviewed by Warrell, 1997). Post-mortem examination of brains of individuals who had died of CM, revealed cerebral pigmentation due to haemozoin deposition resulting from haemoglobin breakdown and some petechial haemorrhages around capillaries. The rest of the brain appeared relatively normal with little extravascular pathology (MacPherson *et al.*, 1985). Mechanical obstruction of cerebral vessels by adherent parasites was also thought as a mechanism causing cerebral malaria. Indeed post-mortem studies have shown plugging of capillaries with infected erythrocytes (Warrell, 1990, Aikawa *et al.*, 1990). Carotid angiography, done on some patients with post-cerebral malaria stroke, however, revealed that only a portion of the patients had large vessel obstruction while the others had clear vessels (Gillo and Warrell, 1993). The

other cases in which no vascular obstruction is found, neurological deficit was thought to be due to cerebral infarction caused by an imbalance between the energy demands of the cerebral tissue, especially during convulsions, and the shortage of energy supply due to hypoglycaemia and anaemia (reviewed by White and Ho, 1992). Another mechanism suggested to play a role in the pathogenesis of cerebral malaria is local nitric oxide (NO) production by cerebral endothelium with a consequent disturbance of neurotransmission (Clark *et al.*, 1992).

Most of the survivors recover fully but some 5% of adults have remaining neurological sequelae (Warrell *et al*, 1982) including psychosis, extrapyramidal features, hemiparesis, neuropathies including the cranial nerves, focal epilepsy and Guillian-Barre syndrome (Gilles and Warrell, 1993), whereas 10% or more of the affected children do show sequelae (Brewster *et al*, 1990). Common sequelae in children include hemiplegia, behavioural disturbances, tremors, mental retardation and epilepsy (reviewed by Warrell, 1997).

In the murine model of CM, *P. berghei* ANKA infection in C57Bl/6 mice leads to paralysis, coma and then death after 6-9 days (Rest *et al.*, 1982). There are differences noticed between the human cases and the murine model of CM. These include, the infiltration of the mice cerebral capillaries with mononuclear cells rather than erythrocytes (Rest *et al.*, 1982, Polder *et al.*, 1988). Also in the murine CM, cerebral oedema has been demonstrated by the reduced brain density and break down of the BBB by Evans blue (Thumwood *et al.*, 1988). Two types of histological changes were reported in *P. berghei* ANKA-infected mice. Type I seen in acute lethal cerebral malaria characterized by widespread microglial activation, endothelial cell damage, and microvascular disruption in the brain tissue. Type II was seen in animals looking normal clinically, characterized by focal mononuclear cell inflammation without endothelial damage. The authors suggested that the same might be true for human cerebral malaria (Jennings *et al.*, 1998).

Recently it was shown that tumor necrosis factor α receptor 2 (TNFαR2) expression on brain tissues is required for the development of experimental cerebral malaria (Stoelcker *et al.*, 2002). Also it was shown that during CM there is increased expression of TNFR2 on brain vascular endothelium (Lucas *et al.*, 1997). Mice deficient in TNFR2 were protected from the lethal syndrome and did not increase expression of ICAM-1on brain microvascular endothelial cells (Erickson *et al.*, 1994)

Malaria-associated problems in pregnancy

The prevalence of low birth weight, spontaneous abortions and stillbirths has been reported to be higher in pregnant women infected with *P. falciparum* malaria than healthy pregnant women (McGgregor, Wilson and Billewicz, 1983, Watkinson, Rushton and Lunn, 1985, Stekette *et al.*, 1996a). Congenital malaria is a rare condition defined as finding parasitizes erythrocytcc in the blood of a neonate of < seven days of age (Djibo and Cénac, 2000). The mechanisms for this are not known but it could be possible that there is trans-placental transfer of the recently-discovered forms, the merophores (Landau *et al.*, 1999).

Other manifestations of falciparum malaria include black water fever, multi-organ failure, coagulopathies, respiratory distress and hypoglycaemia. In most adults who die from falciparum malaria, there is multi-organ failure. The principal causes of death are pulmonary oedema, acute renal failure, metabolic acidosis and circulatory collapse (Gillis and Warrell, 1993).

Factors affecting the disease severity in malaria:

Several factors play a part in determining the clinical presentation of falciprum malaria and its severity. Among these are endemicity of disease, factors inherent in the pathogen and factors inherent to the host.

Disease Endemicity

It has been noted that in holoendemic areas (constant exposure), anaemia predominates in young children, while in hyperendemic areas (seasonal exposure) cerebral disease is commoner (Pasvol *et al.*, 1995).

Pathogen factors

Pathogen-related factors include tendency of parasitized erythrocytes to cytoadhere to endothelial cells and to form rosettes (Pasvol 1992). Studies examining the effect of these factors on disease severity have not produced conclusive results. Details are mentioned under cytoadherence and rosetting.

Host factors:

These include genetic factors such as TNFα promoter polymorphism (McGuire *et al.*, 1994) and haemoglobinopathies, diet, age, sex, pregnancy and parity, social factors such as education, health seeking behaviour and the availability of health facilities both in peace and war (Prusty, 2001). Details will follow under immunity against malaria.

Immunity against malaria:

In malaria endemic areas, it has been reported that immunity against non-cerebral severe disease is acquired at a young age after possibly two attacks of the disease (Gupta *et al.*, 1999). Immunity to the disease in general, however, develops after many years. This state of acquired immunity is established after several years of living in endemic areas and is referred to as premunition (Trape *et al.*, 1994, Bottius *et al.*, 1996) and probably represents a balance between the host and the pathogen, which allows survival of both. The chronicity of infection is thought to be due to several features of the malaria parasite including antigenic polymorphism in different isolates and antigenic variation (Hommel and Semoff, 1988). Recently another mechanism for malaria chronicity was suggested following the discovery of new forms of the parasite hitherto unknown. Impression smears of organs and lymphatics of infected animals showed structures referred to as merophores containing merozoites which have resisted lysis seen with other asexual stage parasites. Their number was found to be proportional to the number of maturing schizonts. The discovering team suggested that they could play a role in the recrudescence and chronicity of rodent malaria. (Landau *et al.*, 1999).

The protection gained by the host is thought to be due to a mechanism involving an interaction between IgG and monocytes referred to as antibody-dependent cellular inhibition (ADCI) (Lunel & Druilhe, 1989, Bouharoun-Tayoun *et al.*, 1990). This mechanism requires the presence of a certain level of merozoites in the circulation to trigger it, hence the persistence of a very low level of parasitaemia. It is also thought to be non-selective for any particular antigenic variants, otherwise the level of parasitaemia would show large fluctuations which is not the case in chronic disease (Druilhe and Perignon, 1997).

Age

The risk of malarial disease is significantly reduced during the first quarter of infancy across a wide range of transmission intensities (Snow *et al.*, 1998). This early protection is attributed to the presence of maternal malaria-specific IgG in the foetal circulation (McGregor 1956), presence of haemoglobin F (Pasvol, 1977) and riboflavin and p-aminobenzoic acid (PABA) deficiency in infants diet (Bates, 1982). During the second quarter of infancy the risk of malaria severity seems to depend on the transmission rate, being high in areas of high transmission. In such areas, however, the risk declines after the sixth month of age, implying that clinical immunity is acquired at a very early age (Snow *et al.*, 1998). McGregor described an antitoxic immunity in the age group of 2-3 years exposed to heavy infection since birth. In this group asexual parasitaemia is high and yet without apparent illness (McGregor, *et al.* 1956). The general consensus is that in sub-Saharan Africa *P. falciparum* predominantly affects children < 5 years old among whom mortality is greatest (Marsh, 1992).

Diet

Perhaps the importance of diet in malaria infection was an accidental finding. It was noticed that rats fed a milk diet were difficult to infect with *P. berghei*. The protective role of milk was subsequently confirmed by Maegraith, Deegan and Sherwood Jones (1952) who reported suppression of parasitaemia in rats infected with *P. berghei* and fed on cow, human or reconstituted dried milk. The reason for this resistance was found to be the lack of p-aminobenzoic acid (PABA) in milk and indeed this suppression was reversed with the addition of PABA (Hawking, 1954).

Later studies showed the involvement of other dietary agents like cod liver oil which was found to prolong survival of *P. berghei* infected mice despite the presence of p-aminobezoic acid in their diet. This suppression was, however, reversed on addition of antioxidants vitamin E, C or tetraethylthiuram disulfide (Godfrey, 1957). The authors

suggested that unsaturated fatty acids of the cod liver oil were oxidized by the host to peroxides creating a hostile pro-oxidant environment to the parasite. Vitamin E, which is an anti-oxidant, prevented peroxidation and so provided parasite friendly conditions. Also, vitamin E deficient diets enriched with fish oils were shown to suppress lethal *P. yoelii* infections in athymic mice and led to survival without developing detectable parasitaemia in SCID/bg mice infected with non-lethal *P. yoelii*. (Taylor *et al.*, 1997).

Restriction of dietary protein intake in mice and rats reduced severity of *P. yoelii*, *P. berghei* and *P. vinkei* infections (Ibekwe and Ugwunna, 1990). It has also been noticed that severe malaria is extremely rare in children with protein-energy malnutrition such as Kwashiorkor and marasmus, and that nutritional supplementaion in malarious patients exacerbates the disease (Marsh, 1995). It is worth mentioning that traditional medicine of some communities involves reducing red meat protein intake and replacing it with fish protein in any acute illness characterized by fever and lethargy including malaria (personal experience, my mother). Furthermore, it was shown that the addition of ω-3-fatty acids of fish origin inhibited the development of *P. falciparum in vitro* (Kumaratilake *et al.*, 1992).

Effect of a combination of diet and red cell abnormalities was seen in the case of eating fava beans in patients with G6PD deficiency. Vicine and convicine constituents of fava beans produce potent pro-oxidants upon hydrolysis. These products increase phagocytosis of malaria-infected erythrocytes especially G6PD deficient cells (Ginsburg *et al.*, 1996).

Pregnancy

During pregnancy, previously immune women become susceptible to malaria infection and tend to suffer severe disease. Primgravida seem to be at a higher risk than mutligravida in both prevalence and severity of disease (McGregor *et al.*, 1983, Fleming *et al.*, 1989, Kasumba *et al.*, 2000). The increased susceptibility to malaria during pregnancy is not due to reduced humoral immunity since no significant drop in the levels of malaria-specific antibodies was found in the state of pregnancy (McGregor *et al.*,

1984). It is thought to be due to the sequestration in the placenta of a subpopulation of isolates which preferentially bind to placental molecules such as chondroitin sulphate A (Fried and Duffy, 1996, Beeson *et al.*, 2000) and hyaluronic acid. Also, Non-immune IgG were recently shown to bind to the Duffy binding-like (DBL) domain of the PfEMP1 molecule on the surface of infected erythrocytes and anchor them to the syncytiotrophoblast through binding to Fc receptors (Flick *et al.*, 2001). Resistance in multigravida, on the other hand, has been attributed to the presence of antibodies against a wide range of placental parasite isolates (Beeson *et al.*, 1999, Freid *et al.*, 1998).

Genetics;

Polymorphism in red blood cells

Involvement of host genetic makeup in susceptibility to malaria was noted by Haldane who suggested that the very high frequency of thalassaemia in the Mediterranean region was not due to high frequency of mutations but rather due to resistance the heterozygous state of thalassaemia confers against malaria (reviewed by Weatherall, 1997). Also, early studies reported that HbS provided protection against *P. falciparum* (Allison, 1954) and subsequent studies confirmed that indeed > 90% of Gambian children with HbAS were protected against severe *P. falciparum* disease (Hill *et al.*, 1991).

The mechanisms of this protection are still not fully elucidated though several hypotheses have been put forward. Among these are, sickling and rapid removal by phagocytosis in the spleen of parasitized red blood cells with HbAS (Luzzatto *et al.*, 1970), reduced ability of parasitized red blood cells with HbAS to grow under low oxygen tension environment (Pasvol *et al.*, 1978) and release of toxic substances to malaria parasite from infected red blood cells with HbAS upon sickling (Orjih *et al.*, 1985). Also, Low rosetting tendency was thought to play a part in protection from malaria in individuals with sickle cell trait. This idea, however, proved to be wrong since HbAS red cells had similar tendency of rosette formation *in vitro* as those with HbAA (Udomsangpetch *et al.*, 1993). While reduced cytoadherence and rosette formation was suggested to be the mechanism

of protection from *P. falciparum* malaria in individuals heterozygous for thalassaemia-β and thalassaemia-α (Udomsangpetch *et al.*, 1993, Carlson *et al.*, 1994).

HLA system:

Studies in the Gambia, West Africa, revealed an inverse correlation between HLA-B53 and disease severity with reduced frequency of HLA-B53 in children with cerebral malaria and in those with severe malaria (Hill *et al.*, 1991). Since erythrocytes do not express HLA molecules, it was thought the protection conferred by the HLA B53 molecule could not be related to the parasite's invasion into erythrocytes. Subsequent studies showed that the HLA-B53 was involved in antigen presentation of the hepatic stage antigen LSA-1 to the $CD8^+$ T cells (Hill *et al.*, 1994). Class II molecules HLA-DRB1and DQB which are common in West Africa but rare in other racial groups were also found to be associated with protection against falciparum malaria with a magnitude similar to that produced by HbAS (Hill *et al.*, 1991, 1992).

Tumor necrosis factor alpha (TNFα) promoter

Polymorphism of TNFα gene promotor region has been reported to play a role in disease severity in *P. falciparum* malaria. Gambian children homozygotes for TNF2 allele, which is a variant of the TNFα gene promoter region, were found to have a relative risk of 7 for death and severe neurological sequelae from cerebral malaria (McGuire *et al.*, 1994). The authors suggested that this could be due to increased levels of TNFα production by TNF2 allele, which was reported to be associated with higher constitutive and inducible TNFα transcription levels (Wilson *et al.*, 1994).

Polymorphism in NOS2 promotor region

Polymorphism in inducible nitric oxide synthase 2 (NOS2) promotor region affecting malaria severity have been described. A 1173 C-T polymorphism was found to be

associated with increased nitric oxide (NO) production and protection against cerebral malaria and severe malaria anaemia in Tanzanian and Kenyan children (Hobbs *et al.*, 2002). Also a 954 G to C polymorphism was associated with mild malaria in Gabon (Kun *et al.*, 1998).

CD36

CD36 is one of endothelial receptors for adhering parasitized erythrocytes (Rogers *et al.*, 1996). It also, however, mediates uptake of non-opsonised parasitized erythrocytes by human monocytes and its activation on monocytes by phagocytosis of infected red cells does not induce the release of TNFα (McGilvary *et al.*,1999). Individuals deficient in CD36 were found to be more susceptible to severe and cerebral malaria (Aitman *et al.*, 2000). The authors suggested that the protective role of CD36 was through increase phagocytosis of infected cells and reduced TNFα production.

Immunity against pre-erythrocytic stages:

Neither the sporozoites nor the hepatic stages cause any clinical disease, nevertheless host immune response develops against them. In fact the amount of immune response against the hepatic stages has been shown to determine the susceptibility of the host to the infection. For example in BALA/c mice it has been shown that resistance and susceptibility against the hepatic stage of two related species, *P. berghei* and *P. yoelii* is determined by the size of hepatic response mounted against each parasite. In the case of *P. yoelii* to which BALB/c mice are susceptible, there is little cellular response and significant histopathological changes within the liver are not observed until after hepatic schizonts have begun to rupture at around 44 hr post-injection of sporozoites. In contrast to this is the development of substantial cellular response and histopathological changes within the liver by 24 hr post-injection of *P. berghei* sporozoites against which the mice are resistant (Khan and Vanderberg, 1991).

Several surface proteins expressed during the hepatic stages have been shown to be the target for an immune reaction. Among these are circumsporozoite protein (CSP), thrombospondin related adhesion protein (TRAP), liver stage antigen-1 (LSA-1) and liver stage antige-3 (LSA-3).

Mature sporozoites in the mosquito's salivary glands and in the host circulation have a major surface protein referred to as the circumsporozoite protein (CSP). It contains three regions, the central region consisting of immunodominant multiple repeat sequences that are species specific. The two flanking regions, I and II, seem to be highly conserved among different species. CSP is secreted from the apical end of the sporozoite and translocated posteriorly during its motility (Stewart and Vanderberg 1988, 1991). Also region II of the CSP has a high degree of structural homology to thrombospondin (Cerami *et al.*, 1992) an adhesion molecule also involved in the process of cytoadherence. Hence it has been suggested that the CSP may be involved in binding the sporozoites to the hepatocytes via this region. Once inside the hepatocytes, substantial amounts of the CSP are shed into the hepatocyte cytoplasm (Khan, Ng and Vanderberg, 1992) and as the parasite develops inside the hepatocytes, the CSP becomes localized around the periphery of the parasite. The importance of these events in parasite development or stimulation of the host's immune response is not known yet. The immune response against this surface protein involves both antibody-dependent and independent mechanisms as demonstrated in both murine and human malaria (Mazier *et al.*, 1988).

Antibody-dependent immunity against pre-erythrocytic stages

In murine malaria, the involvement of antibodies to pre-erythrocytic stages was evident when serum from mice immunized with *P. berghei* irradiated sporozoites inhibited the invasion and development of exo-erythrocytic forms *in vitro* (reviewed by Chatterjee *et al.*, 1996). Similar results were obtained *in vivo* when antibodies recognizing the repeat region of the CSP from mice immunized with irradiated sporozoites of *P. berghei* or *P.*

yoelii protected recipient mice against a homologous sporozoite challenge (Potocnjak *et al.*, 1980, Charoenvit *et al.*, 1991).

Antibodies to other sporozoite surface proteins have also been reported to inhibit invasion into hepatocytes. Among these are antibodies recognizing a 17kDa protein found on the parasitophorous vacuole membrane of hepatocytes or erythrocytes infected with *P. yoelii*. These antibodies were found to inhibit the development of intrahepatic forms of *P. yoelii in vitro* (Charoenvit *et al.*, 1995). In *P. falciparum* thrombospondin related adhesion protein (TRAP) which, is found in the micronemes as well as on the surface of salivary gland sporozoites also seems to be involved in the sporozoite invasion into the hepatocytes (Robson *et al.*, 1995) and in the motility of the sporozoites. Antibodies raised against TRAP inhibited hepatocytes invasion *in vitro* (Rogers *et al.*, 1992a, Muller *et al.*, 1993). Also, antibodies against *P. falciparum* knob-associated PfEMP3 which is expressed on the exo-erythrocytic stages as well as in asexual blood stages have recently been shown to inhibit the invasion of hepatocytes by *P. falciparum* sporozoites *in vitro* (Grüner *et al.*, 2001).

Cross-species protection to the pre-erythrocytic stage has been reported in several studies. Mice immunized with *P. falciparum* sporozoites were protected against *P. berghei* sporozoite challenge (Sina *et al.*, 1995). Sera from these animals recognized circumsporozoite protein 2 (CSP2) protein in both *P. falciparum* and *P. berghei* and inhibited *P. falciparum* and *P. berghei* sporozoite invasion of hepatoma cells *in vitro* (Sina *et al.*, 1995). LSA-3 specific human antibodies exert 100% inhibition of invasion of *P. yoelii* into mouse hepatocytes *in vitro* (Brahimi *et al.*, 2001).

The mechanisms by which anti-pre-erythrocytic stage antibodies protect the host are not clearly defined but more than one mechanism could be involved. Anti-CSP antibodies, for instance, have been shown to inhibit the entry of sporozoites into hepatocytes (Mazier *et al.*, 1987, Nudlemann *et al.*, 1988). Some studies, however, have reported an enhancement of hepatocytes invasion by sporozoites by these antibodies. Low titers of

anti-CSP antibodies of *P. falciparum* (Hollingdale *et al.*, 1988) and of *P. yoelii* (Nudelmann *et al.*, 1988) showed an increase number of hepatic form of the parasite.

Anti-CSP antibodies have also been shown to play a part post-hepatocyte invasion. In *P. falciparum* anti-CSP antibodies were found to interfere with the sporozoite attachment to the hepatocytes membrane and subsequent intrahepatocytic development of the parasite (Mazier *et al.*, 1988). It was thought that since CSP persisted throughout hepatic schizogony then anti-CSP antibodies bound to the parasite could block translocation of the CSP to the parasitophrous vacuole membrane increasing therefore the acidity inside the vacuole with a subsequent lysosomic digestion of the trophozoites hence reduce intrahepatic parasite development (reviewed by Mazier *et al.*, 1988).

Antibody-independent mechanisms

T cell involvement in immunity against pre-erythrocytic stages was demonstrated when Chen induced protection with irradiated sporozoites in B cell deficient mice (Chen *et al.*, 1977). Adoptive transfer of cytotoxic T cell clones recognizing an epitope contained within amino acids 249-260 of *P. berghei* CSP provided a high degree of protection in naive mice against sporozoite challenge (Romero *et al.*, 1989). The protective immunity induced by irradiated sporozoites was shown to be $CD8^+$ T cells-dependent (Weiss, *et al.*, 1988, Schofield *et al.*, 1987). Depletion of $CD8^+$ T cells abrogated protection achieved by vaccination with attenuated vaccinia virus (NYVAC) recombinants expressing the CSP (Lanar *et al.*, 1996). The cellular response in the liver of mice infected with *P. yoelii* was shown to vary according to the method of immunization. Irradiated sporozoites induced a significant increase in $CD8^+$ T cells while live sporozoites led to an increase in polymorphonuclear cells, monocyte/macrophages, B cells and a range of T cells (Faure *et al.*, 1995). The mechanism suggested for this protection by $CD8^+$ cells involves IFNγ (Schofield *et al.*, 1987, Weiss *et al.*, 1992) which is produced by $CD8^+$ T cells upon activation (Weiss *et al.*, 1988). Systemic administration of IFNγ protects rhesus monkeys

against a *P. cynomolgi B* sporozoite challenge (Maheshwari *et al.*, 1986). The sterile immunity observed in mice immunized with irradiated sporozoites is abrogated following anti-IFNγ neutralizing antibody treatment (Schofield *et al.*, 1987b, Seguin *et al.*, 1994). Immunization of mice with *P. berghei* sporozoites induces IFNγ production (White, Jarboe and Krzych, 1994). IFN-γ and IL-1 were shown to inhibit strongly *P. falciparum* sporozoites development in hepatocytes *in vitro* but neither exerts any effects directly which means that they lead to effecter mechanisms. IL-1 was shown to inhibit at a very early stage of infection (Mellouk *et al.*, 1987) and its inhibitory effects was shown to be due to its induction of C-reactive protein (CRP) production by hepatocytes which inhibited *P. yoelii* (Pied *et al.*, 1989). IFNγ produced its inhibitory effects intra-hepatically (Mellouk *et al.*, 1987).

Production of IFNγ, however, could not be the only role of $CD8^+$ T cells in immunity against the sporozoites since the presence of $CD8^+$ T cells was found to be essential in protection against *P. berghei* sporozoites. Mice lacking β2-macroglobulin (do not express MHC class I molecules) and hence are deficient in $CD8^+$ T cell function, failed to be protected against *P. berghei* sporozoite challenge despite the fact that this challenge did stimulate the production of IFNγ along with IL-2 and anti-CSP IgG (White *et al.*, 1996).

The role of $CD4^+$ T cells in immunity against the exo-erythrocytic stages appeared later in literature than that for $CD8^+$ T cells. It was shown that a $CD4^+$ T cells clone which had a cytotoxic activity *in vitro* mediated protection against a *P. berghei* sporozoite challenge (Tsuji *et al.*, 1990). $CD4^+$ T cell clones isolated from mice infected with a synthetic peptide corresponding to the amino terminal segment of *P. yoelii* CSP were found to eliminate liver stage parasites from hepatocytes *in vitro* in an MHC restricted manner which (Rénia *et al.*, 1993) and following their adoptive transfer, could protect naive mice against sporozoite challenge apparently by direct killing (Rénia *et al.*, 1993). Activation of $CD4^+$ T cells in response to CSP of *P. falciparum* in individuals from Papua New Guinea has been observed (Doolan *et al.*, 1994). $CD4^+$ T cells responses against the pre-erythrocytic stages of the parasite does not seem to be restricted to CSP but other

sporozoite proteins could be involved. Active immunization with synthetic linear peptides derived from *P. yoelii* protein PySSp2 (Wang *et al.*, 1996) or PyHep17 (Charoenvit *et al.*, 1995) confers solid protective immunity, which is mediated by $CD4^+$ T cells and is absolutely dependent on IFNγ (Reviewed by Good and Doolan 1999).

Apart form T cells, immunity against exo-erythrocytic (EE) stages involves others cells too. Immunity against sporozoites of *P. berghei* involves infiltrations around infected hepatocytes by neutrophils and macrophages (Jap *et al.*, 1982). The explanation of this infiltration is that after 47 hr, when some EE stages start to rupture, their products leak into the circulation for the infected hepatocytes are permeable and these products attract phagocytes (Meis and Verhave, 1988). Also, it was shown that dendritic cells may have a role in antigen presentation to the $CD8^+$ T cells during the hepatic stages of the *P. yoelii* (Bruña-Romero and Rodriguez, 2001).

NO was shown to play an important role in immunity against intra-hepatic stages of the parasite. Inhibition of NO production led to failure of accumulation of $CD8^+$ T cells around infected hepatocytes (Scheller *et al.*, 1997). The source of NO in Brown Norway rats challenged with *P. berghei* sporozoites was found to be the hepatocytes which expressed increased levels of iNOS (Klotz *et al.*, 1995). The authors also found that higher proportion of infected hepatocytes displayed iNOS activity in immunized rats compared to the naive ones after challenge and that eliminating the liver forms by primaquine significantly reduced the ability of immunized mice to express iNOS. The results therefore suggested that hepatic iNOS expression was dependent upon the presence of attenuated sporozoites. The inhibitory effects of IFNγ on *P. falciparum*- and *P. berghei*-infected hepatocytes *in vitro* was found to be mediated through NO (Green *et al.*, 1990, Mellouk *et al.*, 1994).

It is now believed that protection in mice induced by immunization with irradiated sporozoites of *P. berghei* (Schofield *et al.*, 1987, Seguin *et al.*, 1994) or *P. yoelii* (Doolan

et al., 1996b) is dependent on $CD8^+$ T cells, IFNγ and NO suggesting that activated $CD8^+$ T cells secrete IFNγ which subsequently activates iNOS (Doolan *et al.*, 1996).

Role of the acute phase reactants

Both IL-1 and IL-6 have been shown to inhibit the intrahepatic stages of the parasite directly and indirectly (Mellouk *et al.*, 1987, Pied *et al.*, 1991). Both cytokines induce the synthesis of CRP (Heinrich *et al.*, 1990) which is a major acute phase reactant. CRP was found to inhibit *P. yoelii* sporozoites invasion into hepatocytes *in vitro*. This inhibition was also seen *in vivo* when rats with high serum CRP due to prior treatment with turpentine oil were found to be largely protected against *P. yoelii* sporozoites challenge by not developing the blood stages (Pied *et al.*, 1989).

Immunity against asexual erythrocytic forms:

Innate immunity

Innate immunity to any infection involves various elements of the immune system including the mechanical barriers of the skin and mucous membranes, hostile environment produced by chemicals controlling the pH, phagocytic cells, and other proteins such as those of the complement system. Various studies investigating innate immunity in malaria have been done. Results of some of this work is presented.

Role of macrophages:

Macrophages were found to play an important role in the elimination of asexual blood stage parasites in rodent malaria. Administration of 3 mg of silica (blockades macrophages) to *P. c* AS-infected resistant C57Bl/6 abrogated protection resulting in host mortality and delay in parasite clearance. Silica was injected on days -1 or day 6 post

infection. It was shown that macrophage treatment with silica on day 6 was more efficient than on day-1 in suppressing protection leading to 70 % mortality. On the other hand administration of macrophage activating agents, such as muramyl dipeptide or liposome-encapsulated muramyl dipeptide glycerol dipalmitate resulted in significant survival of susceptible A/J mice (Stevenson *et al.*, 1989). *In vitro* studies have shown that macrophages are capable of killing *P. falciparum* (Ockenhouse *et al.*, 1984, Ferrante *et al.*, 1990). The work done by Ferrante group also confirmed the importance of non-antibody mediated mechanisms against blood stages since the macrophage effectiveness was not affected by the presence of antibodies or complement (Ferrante *et al.*, 1990). Macrophages had also been implicated in antibody-dependent cellular inhibition (ADCI) of *P. falciparum.* It was shown that the ADCI of *P. falciparum* was mediated by factors produced by macrophages such as TNF (Bouharoun-Tayoun *et al.*, 1995).

Role of neutrophils

In vitro studies have shown that upon contact with *P. falciparum*-infected red cells, neurtophils NADPH oxidase becomes activated with the production of oxygen reactive entities which had been reported to be toxic to *P. falciparum, P. yoelii and P. berghei* (Wozencraft *et al.*, 1984, Dockrell and Playfair, 1984). It appears that this toxicity requires the presence of antibodies and that cytokines like TNFα upregulate the expression of Fc receptors on the neutrophils (Ferrante *et al.*, 1990). On the other hand neutrophils have also been implicated in the pathology of malaria. Their sequestration in the brain and other organs is seen in histological slides of rodent malaria. It was found that treating *P. berghei*-infected mice with anti-PMN monoclonal antibodies abolished PMN sequestration in the lung and partially reduced monocyte sequestration in the lung and brain (Senaldi *et al.*, 1994).

Role of dendritic cells

It has been shown that the infected red blood cells actually bind to dendritic cells and by doing so, inhibit their maturation and the subsequent T cells stimulation (Urban *et al.*, 1999). This was thought to be one of the ways the parasite evades the host immune system and results in chronic infection. Recently, however, it was shown that internalization of haemozoin by dendritic cells may actually activate them (Coban *et al.*, 2000) with subsequent activation of specific immunity through activation of T cells and of innate immunity through the production of IL-12 and interferons (1999, Mellman and Steinman, 2001).

Role of natural killer (NK) cells

It was shown in murine malaria that, on their own, NK cells from primed spleens of *P. c.* AS infected C57Bl/6 could not transfer protection to naive mice. NK cell deficiency, however, resulted in higher mortality in deficient mice than controls. This does indicate a role for NK cells in protection albeit not essential (Kitaguchi *et al.*, 1996). It has also been shown that the major expanding lymphocyte population during murine malaria are the intermediate TCR (TCR^{int}) cells, $NK1.1^-$ and $NK1.1^+$ cells (Pied *et al.*, 2000, Weerasinghe *et al.*, 2001) which constitute part of the innate immunity. It was shown that depletion of $NK1.1^+$ cells resulted in a prolonged parasitaemia but the mice did recover from their infection (Mannoor *et al.*, 2001) indicating therefore the role of $NK1.1^-$ cells in resistance against murine malaria.

Role of Complement

Several studies have indicated a role for complement in protection against both human and rodent malaria. Complement was shown to kill both human and rodent malaria parasites at different stages of the life cycle *in vitro* in the presence of specific antibodies (Healer *et al.*, 1997, Pang & Horii, 1998). Complement was also shown to promote *P.*

falciparum killing by the monocytic cell line THP-1 *in vitro* (Kumaratlike *et al.*, 1997). In *P. berghei*-infected rats complement depletion by injection of cobra venom caused rapid and higher parasitaemia and a higher mortality than the controls (Ward *et al.*, 1981). In *P. c. c.* AS-infected mice depletion of C1q (a component of the classical pathway) by gene targeting resulted in a higher peak of primary parasitaemia. More importantly, however, the mice were susceptible to a secondary challenge with the same parasite indicating a role for C1q in the development of immunity to reinfection (Taylor *et al.*, 2001).

Specific Immunity:

In general specific immunity refers to immune responses involving T cells (cell-mediated immunity which is the predominant form against intracellular pathogens and B cells with their immunoglobulins (humoral immunity) which are predominant against extracellular pathogens. Again a wide range of experimental work has been done investigating the role of this arm of the immune system against asexual erythrocytic stages of malaria.

Role of T cells

T lymphocytes are characterized by the presence of the surface receptor $CD3^+$ and are subdivided into 2 main groups according to the surface markers $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are further divided into T helper 1 (Th1) cells producing IL-2, IFNγ and TNF-β and Th2 cells producing IL-4, 5, 6 and 10 upon activation (Mosmann *et al.*, 1986, 1989, Cherwinski *et al.*, 1987)

A rich pool of evidence exists regarding the importance of T cells in malaria immunity. In rodent malaria, the importance of T cell was demonstrated in early experiments using thymectomised animals, which developed a more severe disease than intact animals

(Brown *et al.*, 1968, Weinbaum *et al.*, 1976, Jayawarda *et al.*, 1977, McDonald and Phillips, 1978b). Transfer of immunity to asexual blood stages was achieved by transfer of splenic and lymph node cells from immune animals (Phillips, 1970, Kasper and Aglar, 1973). Moreover, it was reported that the appearance of crisis forms (which represent intraerythrocytic degeneration of parasites) in *P. c* AS-infected resistant C57Bl/6 mice is T cell dependent (Stevenson *et al.*, 1990).

Regarding the part played by different subclasses of T cells, the vast majority of experimental work has pointed out to the importance of $CD4^+$ T cells in immunity against asexual blood stages. $CD4^+$ T cells have been found to increase in the spleens of malarious mice (Weidanz *et al.*, 1990). Their role in particular was demonstrated in experiments transferring splenic $CD4^+$ T cells from immune donor mice and leading to resolution of acute infection in non-immune infected mice (Cavacini *et al.*, 1986, Kumar and Miller, 1990). Furthermore, depletion of $CD4^+$ T cells by monoclonal antibodies in *P. chabaudi* infected-C57Bl/6 mice led to a significantly higher parasitaemia than the control mice and failure to reduce parasitaemia below 20% (Süss *et al.*, 1988).

The importance of $CD4^+$ T cells was initially thought to be for helping in the production of specific antibodies (Brown *et al.*, 1971). Indeed it was shown that *P. chabaudi*-infected C57Bl/6 mice and depleted of $CD4^+$ T cells by monoclonal antibodies early during infection (day7 or 12 post-infection) failed to clear the parasites, while those treated later (day 19) were successful in resolving the infection and that the resolution of infection was associated with the appearance of malaria specific IgG antibodies (Langhorne *et al.*, 1990). Production of specific antibodies, however, could not be the only function of $CD4^+$ T cells since in *P. chabaudi adami*-infected mice, immunity was achieved by adoptive transfer of T cells although the mice were B cell-deficient (Grun *et al.*, 1981, 1983).

Another role suggested for $CD4^+$ T cell was the activation of macrophages through the production of IFNγ (Stevenson *et al.*, 1990). Human macrophages stimulated with IFNγ

have been shown to kill *P. falciparum* asexual blood stages *in vitro* (Okenhouse, Schulman & Shear 1984) by releasing reactive oxygen and nitrogen intermediates (Okenhouse *et al.*, 1984, Rockett *et al.*, 1991).

During a primary blood stage infection of mice with *P. chabaudi chabaudi*, the T helper 1 and 2 (Th1 and Th2) subsets of $CD4^+$ T cells functions appear sequentially. Circulating IFNγ, which is a Th1 cytokine, peaks 2-3 days prior to peak parasitaemia. Th1 cells, therefore, are thought to be important in controlling the primary parasitaemia. $CD4^+$ Th2 cells, on the other hand, seem to be more important later (day 28) when the parasitaemia has declined to sub-patent levels, to clear the infection (Langhorne *et al.*, 1989, Langhorne *et al.*, 1989). This sequence was confirmed in experiments showing that $CD4^+$ T cells obtained from *P. chabaudi chabaudi*-infected NIH mice on days 16 or 20 post infection had Th1 characteristics *in vitro*, while those obtained from re-infected mice had Th2 characteristics (Taylor-Robinson and Phillips, 1992, 1993, 1994a).

Langhorne and colleagues suggested the following scheme of interaction between the Th1 and Th2 cells in acute *P. chabaudi chabaudi* AS malaria. Th1 cells are thought to recognize *P. chabaudi chabaudi* peptides presented in association with MHC class II molecules on antigen presenting cells and are then stimulated to produce IL-2 and IFN-γ (Mosmann & Coffman, 1987). IL-2 and IL-4 act as growth factors for Th2 cells. These cells in turn after encountering antigens help B cells to produce specific antibodies (Mosmann & Coffman, 1987). IFNγ on the other hand upregulates the expression of MHC class II molecules on macrophages and consequently increase antigen presentation and phagocytosis and release of down stream mediators such as TNF-α and reactive oxygen radicals (Nathan *et al.*, 1983). This indicates that the Th1 effects early during infection are mainly non-specific (Langhorne *et al.*, 1989). This was evident from the work of Jarra and Brown who showed that re-infection of mice 1 day after the peak of primary parasitaemia with the same or different strain of *P. chabaudi* AS led to a non-specific protection against the heterologous challange while specific mechanisms became evident when re-infecting later (Jarra and Brown, 1989).

In falciparum malaria in people, it was found that circulating T cells are reduced during the acute attack of malaria and that the reduction correlates with disease severity, being highest in cerebral malaria. The drop affected $CD4^+$ T cells frequency most markedly. (Hviid *et al.*, 1997). The reduction in peripheral T cells is thought to reflect redistribution of T cells into the site of infection.

γδ T cells were also found to play a role in immunity to malaria. γδ T cells were found to increase in the spleens of mice during acute malaria (Bordessoule *et al.*, 1990, van der Heyde *et al.*, 1993, Ho *et al.*, 1990, Roussilhon *et al.*, 1990). In *P. c.* AS-infected mice, it was found that the majority of splenic T cells were αβ in their normal proportions of $CD4^+$: $CD8^+$ T cells, but that there was an 8-10 fold increase in the γδ T cell population (Langhorne *et al.*, 1993). *P. chabaudi*-infected TCRδ chain knock-out mice ($TCR\delta^{-/-}$) cleared primary parasitaemia with a slight delay but had a significantly higher recrudescent parasitaemia than the wild type. Depletion of αβ cells, on the other hand, led to a failure to clear the infection and had a low malaria-specific antibodies level. These results clearly showed that γδ T cells alone were not efficient in providing help for antibody production which is required for the clearance of the infection (Langhorne, Mombaerts and Tonegawa, 1995). γδ cells in *P. chabaudi adami*-infected C57Bl/6 mice proved to be essential for the suppression of infection. γδ depleted mice by monoclonal antibodies were unable to suppress infection although their levels of splenic $CD4^+$ αβ T cells were similar to controls (van der Heyde *et al.*, 1995).

In human malaria γδ T cells (Vγ1, Vγ2) were found to increase in the peripheral circulation of humans during acute *P. falciparum* malaria and remain elevated for weeks following successful chemotherapy (Chang *et al.*, 1992, Ho *et al.*, 1994, Ho *et al.*, 1990, Roussilhon *et al.*, 1990). Moreover, *in vitro* studies revealed that proliferation of human peripheral T cells from malaria non-exposed individuals to erythrocytic stages of *P. falciparum* is dominated by T cells expressing γδ receptors (Goodier *et al.*, 1992, Behr *et al.*, 1992, Goerlich *et al.*, 1991). Cloned γδ T cells were cytotoxic for *P. falciparum in*

vitro (Elloso *et al.*, 1994). Subsequent studies, comparing T cell responses to *P. falciparum* schizont-extracts and live schizonts, revealed that T cell response to parasite extracts is predominantly αβ while live parasites are required for γδ T cell expansion (Waterfall Black and Riley, 1998). This is in agreement with results obtained from the rodent malaria *P. yoelii* infection which had shown that only infection with live parasites causes a γδ cellular expansion in the spleens of BALB/c mice infected with *P. yoelii* (Kopacz and Kumar 1999). γδ T cells from non-immune donors, were recently found to produce TNFα and IFNγ within 18 hr post contact with *P. falciparum*-infected erythrocytes *in vitro* suggesting a role for γδ T cells in the early non-specific immunity against asexual blood stages (Hensmann and Kwiatkowski, 2001).

$CD8^+$ cytotoxic T lymphocytes recognize antigens presented to them by antigen presenting cells in association with MHC class I molecules (Townsend and Bodmer, 1989). Studies investigating their role in immunity against asexual blood stages of rodent malaria have yielded controversial results. Adoptive transfer of $CD8^+$ T cells from immune to non-immune mice had very little impact on the severity of the disease following challenge of the recipients (Kumar and Miller, 1990), and their depletion with the use of anti-$CD8^+$ antibodies had slight or no effect on the course of the disease (Süss *et al.*, 1988, Kumar *et al.*, 1989). Other studies, however, have shown that $CD8^+$ T cell depletion by monoclonal antibodies in *P. chabaudi adami*-infected C57Bl/6 mice resulted in a significantly higher primary parasitaemia than the untreated mice (van der Heyde *et al.*, 1993). The same group, on the other hand, found that β2-microglobulin deficient-mice which lack functional $CD8^+$ T cells resolved *P. chabaudi adami*, *P. chabaudi chabaudi* and *P. yoelii* 17X malaria indicating that $CD8^+$ T cells are not required to suppress these murine malarias (van der Heyde *et al.*, 1993).

In human malaria, several studies have reported increased numbers of $CD8^+$ T cells in the peripheral circulation of patients who have recently recovered from an acute attack of malaria (Hoffman *et al.*, 1984, Troy-Blomberg *et al.*, 1984). $CD8^+$ T cells have been

suggested as playing a part in the immunosuppression observed in acute malaria. Depletion of $CD8^+$ T cells from peripheral PMN cells obtained from individuals recognized as poor responders to soluble malaria antigen preparation *in vitro*, increased their PM proliferation and IFNγ production *in vitro*. No such effect, however, was observed in PMN obtained from individuals responding well to these antigens (Riley *et al.*, 1989).

Apart from their role in protection against malaria, T cells, however, have also been implicated in the pathogenesis in malaria. For example depletion of $CD8^+$ cells prevents cerebral malaria in mice (Hermsen *et al.*, 1997). Also depletion of *P. berghei* ANKA-infected C57Bl/6 mice of $CD8^+$ T cells by monoclonal antibodies protected them from cerebral oedema, respiratory distress and lactic acidosis (Chang *et al.*, 2001). γδ cells were also found to play a pathological role in murine cerebral malaria. Mice depleted of γδ cells by monoclonal antibody treatment and infected with *P. berghei* ANKA did not develop cerebral malaria, while mice lacking γδ T cells from birth ($\delta^{0/0}$) did develop cerebral malaria, indicating the importance of γδ T cells in pathology if present since birth (Yanez *et al.*, 1999). Deletion of T cells bearing Vβ8.1 TCR with the use of monoclonal antibodies protects susceptible BALB/c mice infected with *P. berghei* ANKA from developing cerebral malaria (Gorgette *et al.*, 2002, Boubou *et al.*, 1999).

Role of B cells and Immunoglobulins

The role of B cells varies in different species malarias. B cell-defecient mice and chickens infected with *P. yoelii* and *P. gallinaceum* respectively died of fulminanat malaria (Roberts *et al.*, 1977, Weinbaum *et al.*, 1976). In B cell-deficient mice infected with *P. chabaudi chabaudi*, infection followed a normal course during the primary peak of parasitaemia but the animals were not able subsequently to clear the parasites (Taylor-Robinson & Phillips 1996, Langhorne *et al.*, 1998). In the case of *P. chabaudi adami-*

infected mice, on the other hand, immunity has been reported as being antibody-independent (Grun and Weidanz, 1981).

Several functions have been suggested for B cells and antibodies in protection against malaria. B cells have been implicated in the switch from Th1 to Th2 cells which is necessary as the disease progresses to the clear parasitaemia (Langhorne *et al*, 1989). In *P. chabaudi chabaudi*-infected, B cell-deficient C57Bl/6 mice (either by life long treatment with anti-μ antibodies or B cell knock out mice) the $CD4^+$ T cells retained a predominant Th1-like response to malaria through out the primary infection. Adoptive transfer of B cells to these mice resulted in the development of Th2 response needed to clear the parasitaemia (von der Weid and Langhorne, 1993, Langhorne *et al.*, 1998, Taylor-Robinson and Phillips 1996).

Another mechanism of antibody-mediated immunity demonstrated in *P. chabaudi*. AS-infected CBA/Ca mice is the opsonisation of parasitised red blood cells during crisis period. IgM, IgG1 and IgG2a immunoglobulins were found to increase during this period and enhance phagocytosis of parasitized erythrocytes by macrophages *in vitro* (Mota *et al.*, 1998). In *P. yoelii* infection, however, protection was found to be directly mediated by antibodies and does not require Fc receptors (Rotman *et al.*, 1998).

In the case of human malaria, the role of immunoglobulins in protection against malaria was evident when immunoglobulins from adults living in endemic areas given to children infected with *P. falciparum* malaria transferred protection to them (Cohen, McGregor and Carrington, 1961).

Among mechanisms proposed for antibody-mediated protection in humans is inhibition of invasion of red cells by merozoites. Antibodies against $MSP1_{19}$ fragment have been found in children in natural infections. These antibodies inhibit the secondary processing of the MSP1 protein required for invasion of red cell by merozoites. Antibodies against $MSP1_{19}$ of *P. falciparum* have been shown to inhibit red cell invasion *in vitro* (Blackman

et al., 1994, O'Donnell *et al.*, 2001). The role of these antibodies in natural protection against malaria has been questioned since no correlation was found between their serum levels in Gambian children and protection against the disease (Dodoo *et al.*, 1999).

Another suggested mechanism for antibodies is in the antibody-dependent cellular cytotoxicity (ADCC). It was found that IgG transferred from immune African Adults to *P. falciparum*–infected non-immune Thai patients although protected the recipients, they were not on their own inhibitory to the parasites *in vitro*. The same antibodies, however, did exert inhibitory effect on the parasites growth when normal blood monocytes were added to the culture (Lunel & Druilhe, 1989, Bouharoun-Tayoun *et al.*, 1990).

Among antibodies found in the circulation of individuals living in malaria endemic areas are agglutinating antibodies to parasite-encoded variant surface antigens PfEMP1. These antibodies have been shown to correlate with protection from malaria in Ghanian children (Dodoo *et al.*, 2001, Ofori *et al.*, 2002) suggesting that protection against malaria is at least partly mediated by variant antigen-specific antibodies. A strong correlation was also found between protection against malaria and levels of specific anti-GLURP antibodies (Oeuvary *et al.*, 2000, Dodoo *et al.*, 2000). Immunization of saimiri monkeys with purified complexes of rhoptry-associated protein 1 (RAP-1) and RAP-2 have been shown to confer partial protection against *P. falciparum* infection (Ridley *et al.*, 1990). IgG reactivities to RAP-1 have been found to be inversely correlated with parasite density in Tanzanian children <5 y of age which suggests that immune recognition of RAP-1 is associated with control of parasitaemia (Jakobsen *et al.*, 1996).

Role of IgE

The involvement of IgE in malaria was investigated because of the fact that IL-4 stimulates the B cells to switch from IgM to IgE (King *et al.*, 1993) and because it had been demonstrated that at least in the case of murine *P. chabaudi* AS malaria there was

a general switch from Th1 to Th2 cells (Langhorne *et al.*, 1989) which secret IL-4 (Mosmann *et al.*, 1986). In the case of human malaria, it was found that 85% of donors from highly endemic areas of *P. falciparum* had elevated levels of total and malaria specific IgE in their sera. Also it was found that higher levels were found in patients with cerebral malaria or severe malaria than in uncomplicated malaria (Perlmann *et al.*, 1994, Perlmann *et al.*, 1997). High levels of IgE in severe falciparum malaria were seen to parallel an elevation in circulating TNFα levels and the authors also showed an ability of IgE to induce TNFα *in vitro* from peripheral blood mononuclear cells. The authors suggested that IgE induction of TNFα could also occur *in vivo* and so contribute to malaria pathology (Perlmann *et al.*, 1997) by causing high levels of circulating TNFα which have been implicated in disease severity (Grau *et al.*, 1989).

Role of cytokines:

The role of various cytokines has been studied in various parasite-host combinations with results indicating that cytokine involvement is greatly influenced by the system involved. Roles of TNFα, IFNγ and Il-4 are described in chapters 4, 5 and 6 respectively

Role of TGF-β:

Transforming growth factor β is a member of structurally similar polypeptides referred to as the transforming growth factor superfamily. The bioactive form is a homodimer and there are three isoforms that have been identified in mammals. TGFβ can produce opposite effects on the same cells depending on their environment at the time. Some of its biological functions include regulation of cell proliferation, control of extracellular protein turnover and modulation of cellular responses (Barnard *et al.*, 1990, Massague, 1990). It is secreted by a number of cells including lymphocytes, macrophages and platelets. Among other functions there is the inhibition of IL-2-dependent T cell

proliferation (Kehrl *et al.*, 1986, Wahl *et al.*, 1988), B cell proliferation and immunoglobulin secretion (Kehrl *et al.*, 1986), IFNγ enhancement of NK cell activity (Rook *et al.*, 1986), ablation of the respiratory burst of activated peritoneal macrophages (Tsunawaki *et al.*, 1988) and suppression of NO release (Vodovotz *et al.*, 1993).

In murine malaria it was shown that high circulating levels of TGFβ early during infection were associated with susceptibility of BALB/c mice to *P. c.c.* AS infections with a relative lack of IFNγ and NO, while low levels of TGFβ were seen in resistant mice which exhibited high levels of IFNγ and NO (Tsutsui and Kamiyama, 1999). Also, both circulating levels of TGFβ and its production *in vitro* by splenocytes were found to correlate inversely with disease susceptibility (Omer and Riley, 1998).

Role of IL-10

IL-10 is a pleotropic cytokine that was initially described as a cytokine produced by Th2 cells capable of inhibiting the synthesis of many Th1 cytokines in response to stimulation with antigen or concanavalinA, and accordingly named cytokine synthesis inhibitory factor (CSIF) (Fiorentino, Bond and Mosmann, 1989).

IL-10 can be produced by a variety of cells. It is produced by B cells (Go *et al.*, 1990), cells of monocyte/macrophage lineage (Fiorentino *et al.*, 1991), T helper cells (Howard *et al* 1992, Moore *et al.*, 1993), mast cells (Thompson-Snipes *et al.*, 1991), cytotoxic T cells (Chen *et al.*, 1991) and thymocytes (MacNeil *et al.*, 1990).

The role of IL-10 was studied in *P. yoelii* malaria in C57Bl/6 mice which were infected with either the non-lethal or the lethal strain of the parasite. It was shown that IL-10 was produced during the first week of infection in mice infected with the lethal strain. Splenocytes of these mice also produced IL-10 during the first week of infection in response to malaria antigens *in vitro*. IL-10 was not detectable in the first week in animals

infected with the non-lethal strain of the parasite. The authors suggested that their results agreed with the general concept of Th2 responses early during infection being associated with a lethal outcome (Kobayashi *et al.*, 1996).

In the case of *P. chabaudi chabaudi* AS malaria, it was shown that female IL-10 knock out mice had a more severe disease with a greater hypoglycaemia, hypothermia and weight loss and greater mortality compared to their wild type counterparts during the acute phase of illness. The knock out mice produced more IFN-γ, TNFα and IL-12p40 mRNA than the wild type animals (Li *et al.*, 1999). In experimental cerebral malaria significantly higher levels of IL-10 mRNA were found in the spleens and brains of resistant animals (Kossodo *et al.*, 1997). The above results indicate a protective role for IL-10 in malaria.

In the case of human malaria, it was shown that plasma levels of IL-10 correlated with the severity of *P. falciparum* malaria, with high levels being found in cerebral and severe malaria, while mild malaria was associated with low levels. It was also found that levels fell within 7 days of starting therapy with anti-malarials by which time the clinical features of malaria have disappeared (Peyron *et al.*, 1994).

IL-10 was suggested to downregulate a proinflammatory process in acute falciparum malaria (Ho *et al.*, 1998). PBMC from patients with acute disease were shown to secrete TNFα and IL-6, 2-4 hr post stimulation with malaria antigens, while IL-10 was detected 8hr post stimulation. This suggested that as IL-10 was secreted and increased, it suppressed the TNFα and IL-6. Addition of exogenous IL-10 was found to suppress TNFα and IL-6 mRNA and this suppression was maximum when IL-10 was added in the first 2hr of stimulation (Ho *et al.*, 1998). Moreover, faster parasite clearance rates were found in association with *in vitro* production of IL-10 by acute phase PMBC in response to both liver and asexual blood stage antigens (Luty *et al.*, 1998).

The role of IL-10 in the development of anaemia in malaria caused by *P. falciparum* was studied. Anaemia in malaria has been shown to be due to several factors but not due to a reduction in the level of erythropoeitin which has been found to be normal or even increased (Kurtzhals *et al.*, 1997). The study conducted on African children with severe anaemia, cerebral malaria and uncomplicated malaria showed significantly lower levels of IL-10 in the severe anaemia than the other groups. It was also found that although TNFα was higher in cerebral malaria, the ratio of TNFα to IL-10 was lower in conscious patients with severe anaemia than the other groups. The authors explained this by the fact that TNFα is known to suppress the bone marrow function and since IL-10 is a counter regulatory cytokine to TNFα, then IL-10 should lift up some of this suppression. Indeed IL-10 has been shown to stimulate marrow cells *in vitro* and countract anaemia in mice. Therefore low levels of IL-10 would allow the marrow suppressive effects of TNFα to appear (Kurtzhals *et al.*, 1998).

Recently it was shown that IL-10 may play a role in resistance to *P. falciparum* after eradication of parasitaemia (Kurtis *et al.*, 1999).

Role of IL-12

Interleukin-12 is a disulfide-linked 70kDa (p70) heterodimer glycoprotein composed of a 40kDa (p40) and a 35kDa (p35) subunits. Both subunits together are required to produce the biological effects of IL-12 (Schoenhaut *et al.*, 1992). IL-12 is produced by a number of cells including dendritic dells (Heulfer *et al.*, 1996), neutrophils (Romani *et al.*, 1997), microglial cells (Aloisi *et al.*, 1997) and monocytes/ macrophages which are the major source of IL-12 (Trinchieri *et al.*, 1995). IL-12 was originally identified as NK cell stimulatory factor and shown to have pleotrophic effects on T cells, B cells and NK cells. (reviewed by Trinchieri, 1998).

There is evidence that IL-12 plays a central role in the differentiation of $CD4^+$ Th0 cells into Th1 phenotype (Manetti *et al.*, 1993, Schmitt *et al.*, 1994, Heulfer *et al.*, 1996) with

subsequent production of IFNγ which in turn leads to production of TNFα and reactive nitrogen intermediates (Stevenson *et al.*, 1995). IL-12 is also thought to form a link between innate and specific immunity. Not only does it enhance cell mediated immunity but also influences humoral immunity by inducing isotype switching through both IFNγ-dependent and independent mechanisms (Metzger *et al.*, 1997). IL-12 mediates several biological activities of human T and NK cells, including induction of IFNγ production, and enhancement of NK cell-mediated cytotoxicity (Kobayashi *et al.*, 1989).

In mice infected with *P. chabaudi* AS, IL-12 was shown to be protective against blood stage malaria (Stevenson *et al.*, 1995). In these animals it upregulated extramedullary erythropoeisis in the spleen, preventing therefore fatal anaemia associated with the infection. Also, *in vitro* production of IL-12 by splenic macrophages obtained from resistant B6 mice infected with *P. chabaudi* AS was significantly higher than that produced by splenic macrophages of susceptible A/J mice (Stevenson *et al.*, 1998). Moreover it showed a beneficial effect when combined with chloroquine in the treatment of A/J mice infected with *P. chabaudi* AS. These animals had a more than 15 fold reduction in parasite load and a 100% survival (Mohan *et al.*, 1999).

Role of spleen

In *P. falciparum*-endemic areas, splenomegaly is a characteristic finding in children. It seems to regress with time for it is unusual for the spleen to remain palpable in adulthood (Gillis and Warrell, 1993).

The spleen has been shown to play several roles in malaria. It has been shown be involved in the phenomena of parasite sequestration and antigenic variation because both these phenomena are lost in *P. chabaudi chabaudi* obtained form splenectomised CBA/Ca mice (Gilks, Walliker and Newbold, 1990). Weiss described a vigorous splenic response in *P. yoelii* malaria. He reported branching of fibroblastic stromal cells (which

he termed barrier cells) and their fusion with one another forming extensive irregular membranous sheets providing a variety of barriers. These barriers trapped parasitized erythrocytes and macrophages and hence increased phagaocytosis of the parasitised erythrocytes. In the non-lethal *P. yoelii* malaria, these barriers protected splenic reticulocytes from parasitization while in the lethal infection no such protection was seen (Weiss, 1989). Antibody-coated parasitized erythrocytes are removed by Fc receptor-mediated interactions with splenic macrophages. This function is increased in acute malaria (Lee *et al*, 1989, Ho *et al.*, 1990a). The spleen has also been suggested to be involved in a process called pitting. In this process infected red blood cells are cleared of their parasites without any damage to the red cells. It was observed in *P. falciparum* malaria that during acute infection, the circulation contained red cells rich in ring-infected surface proteins (P155/RESA) but the cells were devoid of any parasites and the spleen was suggested to be responsible for this mechanism of parasite clearance (Angus *et al.*, 1997). Spleen is also a site for extramedullary erythropoeisis. During acute malaria, the red pulp of the spleen expands and splenic erythropoeisis was evident in the first 24hr post infection with *P. yoelii* in BALB/c mice (Weiss, Johnson & Weidanz, 1989). Another role the spleen plays in *P. chabaudi* AS infection is the help in switching into Th2 response required to clear the infection. It was seen that during the primary peak parasitaemia, there is an expansion in the splenic non-B non-T (NBNT) cells which produce IL-4, helping therefore, the Th0 cells to develop into Th2 cells (Helmby *et al.*, 1998). *In P. chabaudi adami* infection it was shown that antibody-independent immunity to reinfection was spleen dependent (Grun, Long & Weidanz, 1985).

Immunity against sexual stages

Studies on the parasite's fate in the mosquito have reported the existence of a number of mechanisms aiming at reducing parasite population inside the vector. Tens of thousands of gametocytes can be ingested into the mosquito blood meal but normally just 50-100 ookinetes are produced, from these typically <5 survive to produce oocyts on the midgut

wall. Of 50,000 sporozoites produced. Only 15-80 might be inoculated by the feeding mosquito into the host (reviewed by Sinden and Billingsly, 2001).

Both antibody-dependent and –independent mechanisms have been described to play a role in immunity against the sexual stages. There are a number of antigens on the surface of the *P. falciparum* sexual forms which can mediate humoral immune response. Antibodies directed against surface proteins of gametes or gametocytes of malaria parasites have been shown to modify infectivity to mosquitoes (Carter *et al.*, 1988). Antibodies to the glutamate-rich repeat region of Pf11.1 in the presence of complement were found to suppress transmission in the mosquito (Feng *et al.*, 1993). Complement mediated-lysis of gametes has also been shown with antibodies to the gamete surface protein Pfs230 (Quakyi *et al.*, 1987, Healer *et al.*, 1997). Other surface proteins that have been found to mediate antibodies capable of blocking transmission include Pfs48/45 (Kaslow, 1993) and Pfs25 which is found on the surface of the zygote and early ookinetes (Kaslow *et al.*, 1991). Antibodies have been shown to block transmission by both interfering with the process of fertilization and sporogony and by antibody mediated complement-dependent lysis of the parasites (Ponnudurai *et al.*, 1987, Read *et al.*, 1994). Also they have been reported to oppsonize the gametes inside the mosquito midgut and enhance their phagocytosis by leukocytes (Lensen *et al.*, 1997).

Antibody-independent mechanisms have been reported for *P. vivax* and are thought to be mediated by TNFα. Reactive nitrogen intermediates (RNI) were found to reduce gametocyte infectivity in mosquitoes (Motard *et al.*, 1993). The components of the alternative pathway in the mouse serum have been shown to reduce but not totally eliminate the infectivity of *P. yoelii* to *Anopheles stephensi*. This reduced infectivity was largely due to the inability of the zygote to transform into the ookinete (Tsuboi *et al.*, 1995).

Al-Olayan and colleagues, found that >50% of *P. berghei* midgut parasites die by apoptosis before invasion (Al-Olayan *et al.*, 2002). It was shown that during the stages of

ookinetes invasion of the midgut epithelium and during invasion of the sporozoites of the salivary glands a set of immune genes was transcriptionally activated both locally and systemically in the fat body (reviewed by Dimopoulos, 2001). In *A. stephensi*, iNOS expression was detected in midgut soon after midgut invasion by the parasite and also later during the release of sporozoites. Circulating nitrates and nitrites were significantly higher in parasite-infected mosquitoes. Dietary supplementation of *A. stephensi* with L-arginine (NO substrate) reduced parasite infection in the vector, while NOS inhibitors increased infectivity (Luckhart *et al.*, 1998). Several antimicrobials were isolated from *A. gambiae*. They are induced by natural and experimental infections. Among them are cecropins, defensin and gambicin. They are effective against bacteria, some fungi and gamibicin is marginally lethal to *P. berghei* (Vizioli *et al.*, 2001).

Diagnosis of malaria

In some countries endemic for malaria local people recognize the disease by its episodic rigors (personal experience). Laboratory diagnosis for malaria, however, is divided into microscopic and non-microscopic tests.

Microscopic tests include examination of blood smears. Thick films are made first to detect parasitaemia. Their sensitivity is of 5 parasitized red blood cells (pE)/µl, while thin films which are made to identify the particular type of parasites have, sensitivity of 20 pE/µl. Microscopic tests have the disadvantages of requiring costly tools like microscopes, electricity and personnel with experience in examining blood films. They are also labour-intensive and time consuming.

During the past ten years, however, there has been the development of rapid diagnostic tests (RDTs) that are based on the detection of antigens derived from malaria parasites in lysed blood, using immunochromatographic strips. They are divided into two groups according to their major *P. falciparum* specific antigen targets, histidine-rich protein 2

(HRP2) and parasite lactate dehydrogenase enzyme (pLDH). Field studies have reported sensitivity of >90% approaching 100% with at high parasite density and specificity of >90%. These tests can be performed in 15 minutes, do not require heavy equipment and do not require long training of the personnel with the consequent advantage of making a rapid diagnosis of malaria and early treatment. The tests, however, are more expensive than microscopy ($ 2-4/ test) and are not quantitative nor can they differentiate between the different parasite species (Wongsrichanalai, 1999).

A novel method for diagnosing malaria is based on detection of haemozoin in peripheral blood monocytes by automated flow cytometry instrument while counting the full blood count. The test is designed to diagnose malaria even in the absence of clinical suspicion of the disease. Field studies in South Africa reported a sensitivity of 72% and specificity of 96%, while in Portugal; sensitivity was reported to be 95%. The disadvantage is that the test requires heavy expensive equipment and therefore is not suitable for poor endemic countries. It is more suitable for areas where physicians do not encounter malaria often and therefore may not suspect it clinically. Another disadvantage is that it can give rise to false positive results due to the persistence of haemozoin containing monocytes in the circulation for 2-3 weeks following cure (reviewed by Hänscheid, 2000)

Control of Malaria

Several factors are responsible for the inability to control the disease including lack of an effective vaccine, resistance of parasites to chemotherapy and insects to insecticides, poverty and civil strife. Poverty is linked with malaria in a vicious circle. Malaria-associated morbidity imposes a huge burden on endemic countries economy, which in turn results in shortage of tools such as antimalarial drugs and bed nets required to combat the disease. Refugees' movement due to civil strife can result in the appearance of malaria in previously malaria-free areas (Greenwood & Mutabingwa, 2002).

In the 1950s and 1960s, WHO had a global malaria eradication project. The project was abandoned due to its high cost and the appearance of DDT resistant mosquitoes (Shiff, 2002). In the late 1990s a new programme (Roll Back Malaria) was set with more realistic objectives for malaria control. The objectives were to reduce malaria related mortality and morbidity to half by 2010 and half it again by 2015 (Nabarro and Tayler, 1998). The programme is a partnership between malaria endemic countries, UN agencies, research community, private sector and media. The technical strategy of the programme is based on early detection and prompt treatment of malaria cases, detection and control of malaria epidemics, mosquito control and prevention of malaria in pregnancy (WHO, Bull, 2000).

WHO also aims to develop sensitive indicators to detect epidemic risks in areas with different levels of epidemicity. Satellites are in use to detect climatic changes favouring mosquito breeding in order to give an early warning to the health authorities in the areas concerned about a potential increase in the frequency of the disease (Rogers *et al.*, 2002)

Several measures could be used to control malaria, these are:

Vaccines

There are two major problems in the development of vaccines against malaria, first the number of different stages that exist in the complex life-cycle of the parasite and secondly the antigenic variation of the asexual blood stages. It follows, therefore, that for a vaccine to be successful it needs to include epitopes from different stages of the parasite's life cycle and to include those epitopes which are more or less highly conserved among different isolates such as the cross-reactive epitopes of PfEMP1 molecule (Gamain *et al.*, 2001) and are immunogenic.

Malaria vaccines are classified into three types according to the parasite stage they target.

Pre-erythrocytic stage vaccines

Several studies have shown a role for pre-erythrocytic stage proteins in protection of the host and hence a potential role in the synthesis of antimalaria vaccine. Irradiated sporozoites elicit protection to challenge with live sporozoites in mice (Nussenzweig *et al.*, 1967), humans (Clyde *et al.*, 1975, Egan *et al.*, 1993) and monkeys (Collins, & Contacos, 1972). The advantage with these vaccines is that there seems to be some cross reactivity between species of the parasite. This was evident in the high protection rate (>60%) achieved in *P. beghei*-infected mice that previously had been inoculated with irradiated sporozoites of *P. falciparum* (Sina *et al.*, 1993). Although complete protection can be achieved with irradiated sporozoites, this method does not seem to be practical because of the large number of infected mosquitoes required for each individual (reviewed by Phillips, 2001).

A chimeric protein consisting of a fusion between the CSP and the hepatitis surface antigen (HbsAg) named RTS,S has gone through a field trial in The Gambia. It resulted in a significant reduction in the rate at which adult males were infected after vaccination (Bojang *et al.*, 2001).

A DNA vaccine including epitopes from six different *P. falciparum* pre-erythrocytic antigens fused to the full sequence of TRAP has been studied in Gambia since year 2000 with encouraging results (Ferry, 2000). Also a recombinant anti-CSP vaccine was found to produce protection to naïve humans (Kestr *et al.*, 2001).

Vaccines against pre-erythrocytic stages are thought to act by inhibiting hepatocyte invasion by the sporozoites or inhibit intra-hepatic development of the parasite, and consequently stop disease development (Richie and Saul, 2002).

Asexual erythrocytic stage vaccines

The potential of blood stage antigens as vaccines was demonstrated in early experiments using crude antigen preparations in animal models (Phillips *et al.*, 1992). Several asexual blood antigens have been used in vaccine development. Immunization with purified rodent malaria MSP-1was successful in protecting mice (Holder *et al.*, 1982). In phase 1 trial of $MSP\text{-}1_{19}$, immunization of non-exposed human volunteers induced high levels of antigen-specific Th1 and Th2 cytokines, the bias of which could be regulated by adjusting the antigen dose and number of immunizations. Since the responses produced to $MSP\text{-}1_{19}$ in the vaccinees were also found in adults living in malaria endemic areas, the authors concluded that vaccination by $MSP\text{-}1_{19}$ could boost the naturally acquired immunity in the semi-immune individuals (Lee *et al.*, 2002). AMA-1 was found to produce protection in monkeys (Collins *et al.*, 1994) and in rodents (Anders and Saul, 1998). A synthetic PfAMA-1 ectodomain was found to induce the production of antibodies that inhibited red cell invasion by *P. falciparum in vitro* (Kocken *et al.*, 2002).

Transmission blocking vaccines

In the rodent malaria *P. berghei* it was found that immunization of mice with Pbs21 (a protein expressed in mosquito midgut stages) could elicit antibody responses that totally blocked the development of parasites in the midgut of mosquitoes (Margos *et al.*, 1993). In the case of *P. falciparum*, several sexual stage antigens (Pfs230, Pfs48/45) and zygote ookinete antigens (Pfs25, Pfs28) have been identified as potential targets for antibody-mediated transmission blocking immunity. Such vaccines, although not beneficial to the host directly, are aimed at blocking effective transmission of the parasites into the mosquito i.e. break the parasite's life cycle and hence prevent transmission of infection into more individuals (Richie and Saul, 2002).

A major difficulty facing production of an effective vaccine against malaria is the short term protection achieved by the vaccines tested so far (Stoute *et al.*, 1998). Several adjuvants have been used to prolong immunity, among these are the *in situ* expression of the vaccine through attenuated strains of *salmonella*, developing chimera of the vaccine and *hepatitis B* surface antigen (RTS,S vaccine) (reviewed by Richie and Saul, 2002) and co-administration of haemozoin with a DNA vaccine encoding Pfs25 which was found to enhance IgG2a responses (Coban *et al.*, 2002).

Chemotherapy

Several classes of drugs are in use against *P. falciparum*. Among these are: -

1) Arylaminoalcohols: derived from the *Cinchona* bark and including quinine, mefloquine and halofantrine
2) 4-aminoquinolines including chloroquine, amodiaquine
3) 8-aminoquinolines such as primaquine
4) Artemisinins: include several semisynthetic derivatives of artemsisin (the active ingrediant of the Chinese herb 'qinghao'), artemether, arteether, and artesunate.
5) Antifolates
6) atovaquone/proguanil
7) antibiotics such as doxycycline, clindamycin and tetracycline (Gilles and Warrell, 1993, Ridley, 2002).

Resistance has been reported to almost all antimalarials (Wellems *et al.*, 2001, White *et al.*, 1998). A recent report regarding sporozoite rates in *An, gambiae s. I* and *An. funestus* in Tanzania has shown a marked increase and this has been attributed to the wide spread chloroquine resistance (Mboera and Magesa, 2001)

Recently a new approach for malaria control has been reported using the currently available drugs. Prophylactic administration of Fansidar at the time of routine

vaccinations in Tanzanian infants reduced the incidence of malaria and anaemia in children substantially (Schellenberg *et al.*, 2001). Also, administration of sulfadoxine/pyrimethamine once during the second and once during the third trimester of pregnancy protected pregnant women against severe anaemia and improved birth weight (Shulman *et al.*, 1999).

The search for new novel drugs to join the armory of drugs against malaria is continuing and several new formulations have been reported to exert a good effect and hence carry good potential. Among these are antihistamines ketotifen and cypoheptadine which inhibit development of hepatic stages of *P. yoelii* (Singh and Puri, 1998). Extracts of *Cassia occidentalis* root bark, *Morinda morondoides* leaves and whole plants of *Phyllanthus niruri* have recently been shown to suppress parasitaemia in *P. berghei* ANKA-infected mice (Tona *et al.*, 2001).

A new class of drugs has been developed which targets membrane synthesis by the parasite. Following invasion of erythrocytes, parasites synthesize membranes essential for their development while such activity is absent from mature erythrocytes. This new class of drugs therefore specifically targets the parasites and has shown promising results. Compound G25 inhibited *in vitro* growth of *P. falciparum* and was 1000 fold less toxic to mammalian cell lines. Also very low doses of the same compound completely cured monkeys infected with *P. falciparum* or *P. cynomolgi* (Wengelnik *et al.*, 2002).

Combination of anti-malarials is useful to decrease the chance of resistant parasites emerging and also to treat patients infected with virulent, resistant strains (Bloland *et al.*, 2000). In parts of south-east Asia for instance, combinations of quinine plus tetracycline and quinine plus doxycyline are commonly used.

Educating mothers how to treat their children correctly and training shopkeepers in the importance of selling a full course treatment both seemed to have produced promising results (reviewed by Greenwood and Mutabingwa, 2002)

Other methods

Several other techniques have been implemented for the control of malaria globally. Among those are the use of bed nets particularly those impregnated with pesticides, covering any sources of still fresh water, regular spraying of houses with insecticides and the use of biological control represented by use of larvivarous fish feeding on the vector larvae in fresh waters (Gilles & Warrell, 1993).

Use of animal malaria models

It would be better if human diseases could be studied entirely in the human population but this is not possible for ethical reasons. In the case of malaria, one is faced with multiple problems including inability to observe the disease progress without medical intervention, inability to check the effect of a possible drug against an untreated control group and difficulties surrounding working with children and pregnant women. Add to this the problem of longitudinal follow up of populations constantly on the move due to civil strife. Another crucial point is the very early phase post infection and before the appearance of the clinical features. This cannot really be studied in the humans since people present to health personnel at variable times post infection. For these reasons, it was essential to conduct studies on animal models which have proved to be extremely beneficial not only in the case of malaria but also in other diseases.

Plasmodium chabaudi chabaudi AS infection in mice has been studies by several groups. It is a reasonable model for the human *P. falciparum* malaria because of a number of features they share. Like *P. falciparum*, *P. c. chabaudi* AS has been shown to sequester in the post-capillary venules (Cox *et al.*, 1987) although with a different site of sequestration. In the case of *P. falciparum*, the asexual stage-infected erythrocytes tend to sequester in the heart, brain, lungs, kidneys, small intestine and liver (Aikawa *et al.*, 1990, Pongponratn *et al.*, 1991) while *P. chabaudi chabaudi* AS-infected erythrocytes preferentially sequester in the liver. Like *P. falciparum*, *P. chabaudi* also infects mature

erythrocytes. *P. chabaudi* infection is also characterized by the occurrence of one or more recrudescence of patent parasitaemia. Finally, both *P. falciparum* and *P. chabaudi* AS exhibit antigenic variation during asexual erythrocytic stage (Mclean, Pearson and Phillips, 1986a).

Aims of project.

This project follows my previous work with Professor Alexander and Dr. James Brewer at the University of Strathclyde. The previous work involved investigating the effects of pre-infection treatment with empty non-ionic surfactant vesicles (NISV) on the weight loss of BALB/c mice infected with *Toxoplasma gondii*. The work also involved measurements of plasma TNFα levels in the NISV-treated and untreated mice. The results of the previous project were promising in that the treated mice lost less weight and had lower plasma levels of TNFα.

Empty NISV were used in this project in *P. chabadui chabaudi* AS-infected BALB/c mice with a number of hypothese to be tested. The hypothese included:

1. To test if pretreatment of *P. chabaudi chabaudi*-infected BALB/c mice with empty NISV had any beneficial effects on the clinical course of the disease including the amount of weight loss in the infected animals.
2. The second aim was to test the effects of pretreatment with empty NISV on the circulating TNFα levels in the treated and infected animals.
3. To determine the mechanisms involved in the above two effects if they were true.
4. To determine if pretreatment with empty NISV had any immunomodulatory effects in the infected animals by measuring the circulating levels of other pro and anti-inflammatory cytokines such as IFNγ and IL-4.
5. Experiments were also performed to investigate the NISV effects on splenocyte proliferation and the production of TNFα and NO in response to various stimulating agents *in vitro*.

6. Finally it was decided to investigate the effect of haemolysis on the measurement of circulating cytokine levels by ELISA. This was achieved by first attempting to measure circulating cytokine levels in whole lysed blood and then to compare them with levels obtained using plasma or serum samples

Chapter 2

Materials and Methods:

Contents

Mice:

Inbred male BALB/c mice were used in most experimental infections. As the place of work was changed during the course of the research, the animals were obtained from two different sources. One group was bred at the Todd Center, University of Strathclyde (Glasgow) and the other group was purchased from Harlan Oak (Bicester, UK). All mice were fed on pelleted CRM(p) Breeder diet (Labsure Ltd). Water was given *ad libitum*. In all experiments, unless otherwise stated, the animals used were 8-12 weeks of age and weighed around 25g. For the mice purchased from Harlan, it was routine to receive them at a younger age (6-8 weeks) and to allow them to acclimatize in the animal house for 1-2 weeks prior to use.

In order to compare the effect of sex on the course of a malaria infection female BALB/c mice were used in some experiments. These again were 8-12 weeks of age, kept in 12 hour artificial light, fed on pellet CRM (p) diet and given food and water *ad libitum*.
For some preliminary *in vitro* experiments, female ICR mice were used

Parasites:

Plasmodium chabaudi chabaudi AS strain was originally isolated from adult thicket rats (*Thamnomys rutilans*) in 1969 by Professor David Walliker (University of Edinburgh) from La Maboke, Central African Republic. The strain was subsequently established in laboratory mice and cloned by the limiting dilution method (Walliker, Carter and Morgan, 1971). Stabilates of parasites, derived from the original AS parent clone, were maintained by cryopreservation in liquid nitrogen and subpassage through mice.

Cryopreservation of blood:

Infected blood, at a parasitaemia of 5-10% containing ring stage parasites, was collected by cardiac puncture from mice sacrificed in a CO_2 chamber into a syringe containing sodium heparin (1000 i.u. /ml) in phosphate buffer saline (PBS, pH 7.2) as an anticoagulant at 10 i.u. heparin per ml of blood. The infected blood was diluted with an equal volume of a sorbitol-glycerol solution (38% glycerol, 2.9 % sorbitol, 0.63% NaCl). 200µl aliquots were dispensed into 1.2 ml cryopreservation vials (Nunc) and labelled with the species of parasite and a code. The vials were snap frozen by immersion into liquid nitrogen and stored until needed.

Maintenance of *P. chabaudi chabaudi* AS

For long term preservation, cryopreservation vials (Nunc) of blood infected with *P. chabaudi AS* parent were kept in liquid nitrogen (-196°C). Infected blood was recovered for experimental use by allowing the vials to thaw at room temperature. Once defrosted, an equal volume of a 17.5% sorbitol solution was added slowly with frequent mixing. The diluted blood was administered by i.p. injection into one or two recipient naïve mice from which the experimental groups were infected once the parasitaemia reached 20-30%.

Challenge infections

The infected blood was obtained from the donor mouse by cardiac puncture. The level of parasitaemia was determined from Giemsa's (Gurr, BDH Ltd) stained thin blood smears. Appropriate dilutions were made with PBS (pH 7.2) to reach a concentration of 5×10^5 parasitised red blood cells (pRBC)/ ml. The recipient mice were placed in a warm box at 30° C for 5-10 minutes to encourage vasodilation. Mice were infected with 1×10^5 pRBCs administered i.v. via the dorsal tail vein as a 200µl inoculum (using a 1ml syringe fitted

with a 26 gauge needle). In case of failure of i.v. inoculation, i.p. inoculations were carried out to minimise stress and its effects on the animals.

Preparation of thin blood smears:

Individual mice were bled by gently piercing the dorsum of the tail with a clean lancet (Sherwood) and spreading the drop of blood formed onto a clean glass slide (BDH Ltd). The slides were fixed in absolute methanol (AnalaR, BDH Ltd) for few seconds and stained in Geimsa's stain (1:10 of Giemsa's stain [Gurr], in phosphate buffer saline, pH 7.2, see Appendix) for 15 min. Following removal from the stain, slides were washed under running tap water for a few seconds to remove excess stain and allowed to dry before examining under the light microscope.

Determination of parasitaemia levels:

The level of parasitaemia of infected mice was determined by daily examination of stained thin blood smears. Samples were taken at approximately the same time of day for each experiment by pricking the tail with a clean lancet. A new lancet was used for each cage. Following staining and fixation, slides were examined under oil immersion using x 100 objective and x 10 eyepiece of a Leitz S. M Lux binocular microscope. Percentage parasitaemia was determined by counting both total RBC and pRBC in a total of at least 10 fields containing around 300 RBC /field. In the time period during the infection when mouse blood was hypocellular (increased plasma: cell ratio) and some of the RBC were haemolysed, as many as possible fields were counted. Means and standard errors of percentage parasitaemia of each group were calculated daily and plotted.

Preparation of empty Non-ionic surfactant vesicles (NISV):

A mixture of 49.6 mg of monopalmitoyl glycerol (Sigma), 16.4 mg of dicetyl phosphate (Sigma) and 46.6 mg of cholesterol (Sigma) was made in a sterile pyrex glass test tube pre-heated for 4 hours at 180° C to minimize the effects of endotoxin. The tube was covered with a piece of aluminium foil and inserted in a heating block at 130°C until melted. Under sterile conditions, 5 mls of warm filter sterile PBS (pH 7.2) were added to the mixture while manually shaking the tube. The tube was covered and immediately transferred onto a vortex (Gallenkamp) for vigorous mixing for one minute. The tube was then left in a shaking waterbath (Gallenkamp) at 60°C and shaking speed of 5 (graded knob) for two hours. A sample was then taken and examined under the light microscope, using x 40 objective lens, to check the morphology of the formed vesicles. The rest of the mixture was transferred into a plastic centrifuge tube and subjected to five cycles of freezing by immersing into liquid nitrogen and thawing by immersing into the water bath. After the final step of freezing, the vesicles were stored at -70°C until further use (personal communication, Dr. J. Brewer).

Administration of NISV:

Prior to use, NISV were allowed to defrost in a waterbath at 60° C at shaking speed of 5 for two hours and then stored at 4°C overnight. 100 µl /mouse of NISV were administered subcutaneously just above the tail.

For short time storage, the vesicles were kept at 4°C for up to a week, after which they were discarded.

Preparation of parasitised and normal red blood cell lysates:

For *in vitro* stimulation of splenocyte suspensions, soluble crude *Plasmodium chabaudi* AS antigens was prepared. The donor mice were kept in a reverse light box (12 hr light between 2000-0800 hr) for about a week prior to blood donation. This was done in order for schizogony to occur during daytime instead of the normal time which is at 0100 hr (Jarra & Brown, 1985). With this system schizogony did occur between 1100-1300 hr and mice were sacrificed at peak parasitaemia and bled through cardiac puncture into heparinised PBS (10 i.u./ml) at 0800-0900 hr, when most parasites were at the late ring stages. After washing twice in 5% FCS-supplemented Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco) at 250g for 5 minutes, erythrocytes were resuspended to a 10% haematocrit in the same medium and cultured using the candle jar method (Trager and Jensen, 1976). 15ml of the 10% v/v suspension of the infected blood in medium were dispenced in 10mm Petri dishes (Greiner) which were placed together with a candle in a humidified glass dessicator.The candle was lit and the lid put on with the stopcock open. When the candle flame extinguished the stopcock was closed. This procedure provided a gas phase of 3% CO2 and 15-17% O2 (Trager, 1987). The candle jar was placed in a 37°C incubator until the parasites had reached the schizont stage; parasite maturation was monitored every 30-45 min by examination of Giemsa's stained thin blood smears. The ccultured parasites were washed in 5% FCS RPMI 1640 medium at 200g for 10 minutes and re-suspended to the original volume in sterile PBS (pH 7.2). The suspension was filtered through a sterile Whatman CF11 powdered cellulose paper column to remove leukocytes (Beutler *et al.*, 1976) and the filtrate was subsequently washed. The pellet containing the malaria parasites was restored to its previous volume in PBS (pH 7.2) and then freeze-thawed five times by snap freezing in liquid nitrogen and immediately defrosting in a 37°C water bath (Gallenkamp). The disrupted parasitized red blood cells were centrifuged at 1500g for 10 minutes and the supernatant was collected. This was labeled as pRBC lysate and stored in 20-50µl aliquots at - 20°C until use. The above procedure was also used to prepare lysate from non-infected erythrocytes excluding culture of cells for few hours. This lysate was termed nRBC and stored as above.

Determination of protein concentration:

The total protein concentration of both pRBC and nRBC lysate was determined by an adaptation of a method described by Smith *et al.,* (1985). Standards of known protein concentrations were prepared by diluting 2mg/ml stock BSA standard (Pierce Chemical Co) in PBS (pH 7.2) to give a range from 1-25µg/ml. Dilutions of unknown protein lysates were prepared in PBS (1:100, 1:1000, 1:10000). 100 µl/well of standards and samples were added to a 96 well plate (Immulon^R 4HBX, Dynex). 100µl of Coomasie Protein Assay Reagent (Pierce) was added to each well. The plate was read at 570 nm on an MRX plate reader and the protein concentration was determined by plotting the results against the graph of known protein standards using Biolinx software (Dynatech).

Collection of plasma samples:

Mice were warmed in the warm box for 5 min to encourage vasodilation. Up to100 µl blood samples were collected from the tail vessels. At certain time points, however, two animals were sacrificed from each group and blood was obtained by cardiac puncture.

Variable degrees of disease severity were noted among animals of the same group at any time point, with some animals being very ill and others looking relatively well. To minimize errors resulting from picking these outliers, samples were collected from a number of animals representing the predominant degree of disease severity across the whole group at each time point. In order to obtain plasma from blood, blood was collected in Eppendorfs containing sodium heparin [(1000 i.u./ml) in phosphate buffered saline (PBS, pH 7.2)] as an anticoagulant at 100 i.u. heparin per ml of blood. Samples were centrifuged at 9000g for 5 minutes and plasma was pooled for each group into Eppendorfs and frozen at – 20°C until use.

Collection and storage of blood samples for cytokines:

Animals were bled through either cardiac puncture or from the dorsal tail vein as described above and the blood samples obtained from animals in the same group were pooled. 100µl of de-ionized distilled water (ddH_2O) were added to every 1ml of whole blood (in order to haemolyse the blood) and stored at -20°C until further use.

Collection of serum samples:

Animals were sacrificed in CO_2 and bled by cardiac puncture. Approximately the same amount of blood was obtained from each mouse and samples were pooled for each group. The blood was allowed to clot, loosened from the edges of the container and allowed to contract overnight at room temperature. The serum was harvested and the contaminating erythrocytes were removed by centrifugation for 5 min at 9000g. Samples were then stored at -20°C until use.

Cytokine ELISAs using plasma samples:

Plasma samples were collected as described previously and assayed for the presence of cytokines TNFα, IFNγ, IL-10, and IL-4. Capture monoclonal anti-antibodies (PharMingen) were diluted to the required concentration of 2µg/ml in coating buffer (sodium bicarbonate buffer, pH 8.2-see Appendix). 50 µl of capture antibody per well were added to ELISA plates (ImmulonR 4HBX Dynex) and incubated overnight at 4°C. On the following day, the plates were washed carefully (allowing at least 30 seconds for each wash and minimising bubbles) three times with 0.05% Tween 20 (Organics, BDH Ltd), PBS, pH 7.2 washing buffer (see Appendix 1). After the final wash, the plates were pounded on dry paper towels to remove any excess antibodies. 200 µl of blocking buffer (10% heat inactivated FCS in PBS, pH 7.2) were added to each well and plates allowed to

incubate for 1 hr at 37°C to block any non-specific binding sites. The plates were washed three times as described above. Fifty µl of appropriate standards (PharMingen) and samples were added to each well. In the case of supernatants of spleen cell cultures, neat concentrations were used as samples while in the case of blood and its derivatives and due to their small available amounts, dilutions were first made in blocking buffer. Following 2-3 hr incubation at 37°C, the plates were washed 4 times and dried as described previously. Fifty µl of secondary biotinolated secondary monoclonal antibody (PharMingen) diluted in blocking buffer to 2µg/ml were added to each well and plates were incubated for another hour at 37°C. Following this incubation step, plates were washed 6 times and dried before 100 µl/ well of streptavidin-peroxidase (SABU) were added and plates incubated for a further 1 hour at 37°C. The plates were now washed 8 times and dried as above before 100 µl /well of 3,3',5,5'-tetramethylbenzidine (TBM) substrate (KPL) warmed to room temperature were added. The plates were incubated at room temperature while wrapped in aluminum foil to avoid light for at least twenty minutes or until the reaction had fully developed. The plates were read at 630 nm with a reference filter at 405 nm on an MRX plate reader (Dynatech). The results of the unknown samples were calculated against a standard curve of known concentrations using Biolinx software (Dynatech).

Measurement of Weights of Animals:

Individual mice were weighed daily at approximately the same time and using the same scales for each experiment. Individual mice were identified by drawing circular bands around their tails using a permanent marker pen (Sanford). Each mouse was briefly covered with a known weight beaker while on the scale to minimize fluctuations resulting from movement. Percentage of weights was obtained by dividing the means of daily weights by the means of weights on day zero (day of infection).

Clinical monitoring of animals:

Mice were examined daily and the degree of their activity and self hygiene was noted for each individual mouse. A scoring system was developed to quantify the health status of each mouse. The means of health scores of all mice in each group were obtained and plotted in a graph.

The following score was used to quantify animals health status.

Hassan Health Status Score (HHSS):

CF/ score	1	2	3	4	5
pilo erection	severe	mild-moderate	nil		
activity	nil	occasional movement	walking slowly/dragging	walking fast	climbing/running fast
back	hunched	straight			
perianal area	dirty	clean			
eyes	shut	partially shut	open and bright		
extension	head down	sniffs/curious	neck only	½ body	full, frequent
urine	bloody/dark	normal			
rate of chest wall movement	slow	rapid	normal		

The rate of chest wall movement was included in the score and taken to represent a combination of both heart rate and respiratory rate. This is because it was difficult to

separate between the chest wall expansion representing respiratory rate and movement due to hyperdynamic circulation of the rapid heat rate. Had a Pulsoximeter been available, it would have been possible to measure the heart and the respiratory rate separately. The rate of chest wall movement ranged from a very rapid rate (180 or >/min) to very slow (20 or </min). Certain features were used as indicators for dying including very slow chest wall movement, which clinically represents exhaustion and the head held down with the eyes shut. Also some features such as urine output (+/- haematuria) and bowl function were observed initially but had to be omitted subsequently because they could not always be observed during the time period of attending the animals.

Splenocyte cultures:

Spleens were obtained aseptically from the sacrificed mice and dissociated by pressing them with a 5 ml syringe plunger through a sterile stainless steel tea strainer to make a single cell suspension in RPMI 1640 medium (Gibco) supplemented with 10% sterile heat-inactivated foetal calf serum (FCS) referred to as complete medium. The cells were centrifuged at (280g) for 5 minutes. To remove the erythrocytes 3ml of freshly prepared Boyl's solution (Tris, Boehringer Mannheim, NH_4Cl AnalaR, BDH, 1:9, see Appendix) were added and allowed to stand for 3-5 minutes at room temperature for red cell lysis to occur. The cells were again centrifuged as above and the supernatants removed to wash off the Boyl's solution. The pellet was re-suspended in 5 ml of complete medium and washed twice more at 1500 rpm (280g) for 5 minutes. After the last wash, cells were suspended in 2ml of complete medium and counted with the use of a haemocytometer and trypan blue (Gurr, BDH Ltd).

The cell suspension was finally diluted to $5x10^6$ cells/ml in complete medium and 100µl containing $5x10^5$ cells were added to each well of a 96 well flat bottomed tissue culture plates (Iwaki). 100µl of complete medium or antigen were added to appropriate wells to

give a total volume of 200 µl /well and the plates were incubated at 37° C in 5% CO_2 in air for various lengths of time according to the assay done.

Proliferation assay:

Cell suspensions were prepared as described and adjusted to a final concentration of 5 x 10^6 cells/ml in 10% FCS RPMI 1640 medium (Gibco). 100 µl of cells/well were aliquoted into a 96 well flat bottomed tissue culture plate (Iwaki) with 100 µl of 10% FCS RPMI 1640 medium, or a range of stimulants including ConA, pRBC lysate, nRBC lysate, pRBC and nRBC. The plate was incubated at 37° C, 5% CO_2 in air for 72 hours. Each well was pulsed with 0.5µCi of ^{3}H-methyl-thymidine (1µ Ci /ml, specific activity 5Ci /mmol, Amersham) in a 10 µl volume of 10% FCS RPMI 1640 medium and the plate was incubated for a further 18 hours at 37° C. The wells were harvested with a semi-automatic cell harvester (Titertek, Flow) onto glass fibre filter paper (FG /A Whatman) and air dried. Each filter disc corresponding to a specific well was transferred to plastic β vials (LKB) and 2ml non-aqueous scintillation fluid (Optiscint safe, LKB) added. The β activity present in each sample was detected during 1 minute using a scintillation counter (LKB Wallac 1219 Rackbeta) and the counts per minute (cpm) calculated using the Ultroterm III software package. All samples were set up in triplicate to enable an arithmetic mean of the cpm, representative of the proliferative response, to be calculated.

Culture of liver macrophages:

Livers were obtained from naive mice under sterile conditions. They were dissociated with the plunger of a 5 ml syringe by pressing through an autoclave-cleaned metal tea strainer in 5 ml of RPMI 1640 (Gibco) supplemented with 10% FCS (this is referred to as RPMI complete medium). The suspension was washed at 600g for 5 minutes once and the supernatants discarded. The pellet was re-suspended in 2-3 ml of freshly prepared

Boyl's solution (1mlTris: 9ml ammonium chloride) and washed again at (600g) for 5 mins. After 2 further washings the pellet was finally suspended in a known volume of the complete medium (20ml) and transferred onto a 10 mm petri dish (Greiner). Following 2 hour of incubation at 37°C in 5% CO_2 in air, the supernatants and any floating cells were discarded, while the adherent cells were scraped off the bottom of the dish with a plastic Pasteur pipette and washed once at 600g for 5 mins. Finally the cells were resuspended in 2 ml of complete medium and counted with in a haemocytometer (Improved Neubar). The final dilution of $1x10^5$ cells / 600µl/ well was made and the cells were plated in 24 well flat-bottomed plates (Iwaki).

Preparation of Macrophages from bone marrow:

Bone marrow-derived macrophages were obtained from a culture of bone marrow cells collected from the femurs and tibiae of naïve mice. The bones were removed aseptically and the distal ends of the femurs and the proximal end of the tibiae were cut. A 26 gauge needle was inserted into the opposite ends (proximal end of femur and distal end of tibia, since in adults blood cell formation occurs at distal ends of femurs and proximal ends of tibiae while the rest of the marrow is replaced with adipose tissue) and the marrow was flushed out with 5 ml of ice cold Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 100mg/ml streptomycin, 100U/ml penicillin, 50 mM 2-b-mercaptoethanol, 20% HI-FCS, 2mM L-glutamine, and 1mM pyruvate (Sigma)). The cells were centrifuged at 600g for 10 mins at 4°C and re-suspended in DMEM supplemented with 30% supernatant of L-929 cells and 20% HI-FCS. The cells were transferred to a 100 mm tissue culture dish (Greiner) and incubated at 37°C in 5% CO_2 in air for 7 days. The non-adherent cells were discarded and the adherent cells were collected by rinsing the dish with ice cold filter sterilized PBS for 10 minutes and also scraping the bottom of the dish with a plastic Pastuer pipette. These cells were centrifuged at 600g for 10 minutes and re-suspended in complete DMEM. A sample was removed to count the cells which were then adjusted to a concentration of $5x10^6$/ml.

Following incubation overnight, the non-adherent cells were removed and fresh complete DMEM (Dulbaco modified Eagles medium, Gibco) was added along with the antigens to be tested. The final volume of each well was 200µl. The cells were then left to incubate for either 3 or 6 hr and the supernatants were collected to assay for TNFα and NO. The samples were stored at –20°C until further analysis.

Determination of nitric oxide production:

The level of nitric oxide production *in vitro* was determined by the measurement of the end product, nitrite, using the Griess reaction described by Migliorini *et al.*, (1991). Nitrite standards were prepared by diluting 0.1g of the stock powder of sodium nitrite (Fisons) in 10ml of de-ionised distilled water. The mixture was diluted to a final concentration of 10µg of sodium nitrite /ml of 10% FCS DMEM medium. The concentration of the standards ranged from 10-0.156µg /ml nitrite. Fifty µl of freshly prepared Griess soultion (1:1 mix of 0.1% (N-1-napthyl)ethyl-enediamine, (Sigma) and 1% sulphanilamide, (Sigma) in 5% orthophosphoric acid, Fisons) were added to 50µl of culture supernatant or standard in an individual well of a 96 well plate (Immulon^R 4HBX, Dynex). The plate was incubated for 10 minutes at room temperature and read at 540nm on a MXR plate reader. The concentration of nitrite for the unknown samples was calculated against a plot of a standard curve using Biolinx software (Dynatech).

(3-(4,5-dimethylthiazol-2-yl)-2.5 diphenyl tetrazolium bromide) MTT assay:

To each well containing $5x10^6$ cells /ml, 50µl of 1mg/ml of [3-(4,5-dimethyl thiazo-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), (Sigma) were added and the tissue culture plate was incubated in 5% CO_2 in air for three hours. 150µl were removed from each well and 100µl of propane-2-ol (Fisons) were added and mixed vigorously. The plate was read at

450 nm using an MRX plate reader and the results were calculated against a standard curve of known concentrations using Biolinx software.

Cell viability counts

The viability of cell preparations was determined by the trypan blue exclusion method. A 20µl volume of 0.2% w/v trypan blue (Gurr, BDH Ltd) in PBS (pH 7.2) was added to a 20µl sample of the cells in PBS (pH 7.2) and mixed thoroughly. 20 µl of the mixture was swiftly pipetted onto an improved Neubar haemocytometer (ARH) and the cells were counted under phase contrast (x 400) on a light microscope. Viable cells remained clear while the dead cells were unable to exclude the dye and stained blue. Five squares (four at each corner and the central square) were counted and the proportion of dead to live cells gave the percentage viability. From these calculations, appropriate cells concentrations for different experimental procedures were derived.

Maintenance of L-929 Cells:

L-929 cells were a kind gift from a colleague (Department of Materia Medica, University of Glasgow) and cultured in sterile flasks (Iwaki) in complete DMEM (Gibco) supplemented with 20 % FCS at 37° C in 5% CO_2 in air. Every fourth day the supernatants were collected into sterile universals and stored at - 20° C until use while fresh medium was added to the cells for further culture.

Statistical analysis:

Results of weights, levels of parasitaemia and health score are expressed as means of the groups +/- standard errors. Groups data were compared using Student's *t* -test. Results were considered significant when the p value of < 0.05 was obtained. Results of levels of

cytokines are expressed as means as measured by the ELISA reader. No standard errors are available since samples from each group were pooled together to allow measurement of 4 different cytokines plus nitric oxide.

Chapter 3

Effects of pre-infection administration of non-ionic surfactant vesicles (NISV) on the course of *P. chabaudi chabaudi* AS infection in male BALB/c mice.

Introduction

As mentioned previously, the aim of this project was to investigate the effects of preinfection treatment with empty non-ionic surfactant vesicles (NISV) on the course of *P. chabaudi chabaudi* AS in BALB/c mice. The rationale behind using NISV was based on an observation made while using empty NISV in mice infected with *Toxoplasma gondii*, details of which are given below.

Non-ionic surfactant vesicles

Non-ionic surfactant vesicles (NISV), also called nisomes, are amphiphilic liposome-like vesicles made of non-ionic surfactants (reviewed by Conacher, Alexander and Brewer, 1998). NISV as used in the present studies were initially made and used by their investigators (Brewer and Alexander, Strathclyde University) to examine their potential for replacing liposomes as adjuvants in vaccines. The need to replace liposomes as adjuvants arose from the fact that liposomes had several disadvantages, which required to be dealt with. Among the disadvantages associated with liposomes is that they enhance the immune response modestly. This problem could be overcome by formulating liposomes in combination with other adjuvants such as of lipid A, aluminium salts, avridine and IL-12 (reviewed by Conacher, Alexander and Brewer, 1999). Liposome encapsulated synthetic antigens containing repeat sequence epitopes from the CSP of *P. falciparum* were shown to induce a strong humoral immune response in rabbits and monkeys (Alving and Richards 1990). Liposomes had another problem relating to one of their components, the phospholipids, which degrade easily in air and require special storage conditions and hence makes the liposomes expensive. In view of the above disadvantages associated with liposomes, research continued for adjuvants better than liposomes. Since the adjuvant action of liposomes and other adjuvants is related to their surface activity (Gall, 1966), which in turn is related to their vesicular structure, it was, therefore, thought reasonable to make vesicles from other compounds such as non-ionic surfactants.

Vesicle formation by non-ionic surfactants has been demonstrated for a number of surfactants such as polyglycerol alkethers, crown ethers, polyoxyethylene alkether/esters, alkyl glycosides and didodecyldimethylammonium derivatives of cholesterol (Brewer, 1993). The vesicles produced were unilamelar or multilamellar structures and displayed many desirable features of liposomes. Moreover, these vesicles exhibited several advantages over the liposomes. They lacked toxicity (Hunter, Strickland and Kezdy, 1981) and were devoid of phospholipids -with their costly purification procedures and storage requirements- making them, therefore, 10-100 times cheaper than liposomes (reviewed by Cochrane, Alexander and Brewer, 1999).

The NISV used here were made from a mixture containing cholestrol, monopalmitoyl glycerol and dicetyl phosphate (details are given in Materials and Methods). Their manufacturers (Brewer and Alexander, Strathclyde University) initially investigated their adjuvant activity in BALB/k mice vaccinated against *Toxoplasma gondii*. In their experiments, toxoplasma soluble tachyzoite antigen (StAg) was entrapped inside the NISV and administered to BALB/k mice subcutaneously on days -28 and -14. A group of mice was also injected but with empty NISV to act as a positive control. All mice were infected with *T. gondii* on day 0 and subsequently followed up with respect to weight loss. An interesting observation made in these studies was that the infected and empty NISV-treated mice lost less weight than the untreated, infected group. The authors suggested that perhaps the NISV led to a reduced TNFα production by macrophages in response to the infection and hence the treated mice lost less weight than the untreated group (Brewer, personal communication). Their suggestion was based on the studies reporting the positive correlation between circulating TNFα levels and cachexia (Beutler *et al.*, 1985).

The aim of this project was to investigate the effects of pre-infection treatment with NISV on animals' weights in malaria and to investigate the above hypothesis regarding the mechanisms involved in the actions of NISV by measuring the circulating levels of TNFα. In the work described here, empty NISV were administered following the same protocol used by the makers of the NISV (Brewer and Alexander, Strathclyde University) to study their effects on the clinical course of male BALB/c mice infected

with *P. c.* AS. The clinical parameters followed up are, weight, percent of parasitaemia and mortality. A health status score was developed to follow up the animals with regards to their degree of activity, appearance and hygiene (Material and Methods, HHSS).

Results

3.1. Effects of NISV on the weight loss of mice infected with *P. c. c* AS

The work presented here covers four experiments conducted on male BALB/c mice at two different venues. The first two experiments (S.1 and S.2) were conducted at Strathclyde University while the last two (G.1 and G.2) were conducted at Glasgow University.

Inbred male BALB/c mice (n = 10, 20, 20 and 25 in experiments S.1, S.2, G.1 and G.2 respectively) were injected subcutaneously with 100µl of PBS or NISV on days -28 and -14 pre-infection and infected with $1x10^5$ pRBC i.v. on day 0 and were designated as groups Pc and PcV respectively (see protocol of NISV administration). Two control groups referred to as PBS and NISV containing 10 animals each (in all experiments) were injected subcutaneously with 100µl of PBS or NISV respectively on days -28 and –14 (Table 3.1). Animals were followed up daily at approximately the same time for each experiment and measurement of the weight of each mouse was recorded using the same scales for each experiment. In order to obtain weights as accurately as possible, each mouse was briefly covered with a beaker while on the scales to minimize its movement.

Table 3.1: Protocol of NISV administration.

Day post infection	PBS	NISV	Pc	PcV
-28	100µl PBS	100µl NISV	100µl PBS	100µl NISV
-14	100µl PBS	100µl NISV	100µl PBS	100µl NISV
0			$1x10^5$ pRBS	$1x10^5$ pRBC

PBS, NISV: groups injected with 100µl of PBS or NISV respectively on day -28 and -14, Pc, PcV: groups inoculated with 100µl of PBS or NISV respectively on days –28, -14 and infected with $1x10^5$ pRBC on day 0.

The initial results of weight loss in experiment S.1 (n = 10) showed a less weight loss in the infected, NISV-treated group (PcV) than the infected and untreated group (Pc). Similar results were obtained in experiment S.2 in which a larger number of animals was used per group (n = 20) (see Appendix 2 for results of individual experiments). Both these experiments were conducted at Strathclyde University.

The difference in weight loss between the two groups (Pc and PcV) was significant only for a certain period post-infection. The duration and timing of this period, however, was not the same in the two experiments. It was from day 10-15 ($p<0.05$, at CI of 95%) and day 7-11($p<0.05$, at CI of 95%) in the first (S.1) and second experiment (S.2) respectively. Peak parasitaemia in both experiments occurred on day 9 post-infection. In experiment S.1 one mouse in the Pc group developed gross oedema from day 16-22 post-infection. The mouse was taken out of the group of the measurements in order to reduce the impact of this outlier. With the data from the two experiments combined, it was seen that significantly less weight was lost in the infected, NISV-treated group (PcV) on day 6 and days 8-11 ($p <0.05$, CI = 95%) than the infected, untreated group (Pc) (Figure 3.1).

On repeating the experiment subsequently at Glasgow University in order to collect enough blood samples for measuring immunoglobulin profile of the mice, the results on weight loss and indeed other parameters did not agree with the previous work. In the first (G.1) of two experiments conducted at Glasgow University, there was a significant difference in weight loss on day 8 only but in favour of the NISV-untreated group (Pc) ($p < 0.05$ at 95% CI). The peak parasitaemia occurred on days 8 and 9 in the NISV-treated and untreated groups respectively.

It was thought that perhaps the NISV used in the above experiment were not made properly and hence led to the contradictory results. The experiment, therefore, was repeated for a fourth time with the NISV being supplied by Dr. J. Brewer, Division of Immunology, Infection & inflammation, Western Infirmary Glasgow. In this experiment (G.2), the infected, NISV-treated group (PcV) had significantly higher weights on days 6 and 10 than the infected, untreated group (Pc) but the latter group had higher weights on days 8, 13, 14 and 15 post-infection than the treated group (PcV) ($p < 0.05$ at 95% CI) (see Appendix for results of individual experiments). Peak parasitaemia was on day 9 and 10 in the untreated (Pc) and treated (PcV) groups respectively. With the two experiments combined, it was seen that a significant difference in percentage weight occurred on day 14 only post infection ($p < 0.05$ at 95% CI) but in favour of the infected, untreated group (Pc) (Figure 3.2).

In summary, in the first two experiments conducted at Strathclyde University (S.1 and S.2), the infected, NISV-treated group (PcV) lost less weight than the infected, untreated group (Pc), while in the second two experiments conducted at Glasgow University (G.1 and G.2) the infected untreated group (Pc) lost less weight than the infected, NISV-treated group (PcV). Weights of the PBS and NISV groups in each experiment were similar.

3.2. Effects of pretreatment with NISV on level of parasitaemia:

The course of the parasitaemia was followed in infected mice in all the above four experiments. Blood smears were obtained from five mice per group in experiment S.1 and from all surviving mice in the subsequent experiments. Smears were stained and fixed and percent parasitaemia calculated as described in Chapter 2. In the first two experiments, conducted at Strathclyde University, percent parasitaemia in the infected, NISV-treated group (PcV) was significantly lower on day 9 and days 5 and 6 in S.1 and S.2 respectively ($p<0.05$ at 95% CI) (see Appendix 2 for individual experiments). With the two experiments combined the NISV-treated group (PcV) had a lower parasitaemia than the untreated (Pc) group from day 5 to day 10 post-infection but the difference was not significant at any point (Figure 3.3).

The results of parasitaemia in experiments conducted at Glasgow University did not agree with those obtained from experiments conducted at Strathclyde University. In experiment G.1, a significantly lower level of parasitaemia was seen in the untreated group (Pc) than the treated group (PcV) on day 8, while in G.2 a lower parasitaemia was seen in the NISV-treated group (PcV) compared with the untreated group (Pc) on days 7 and 9. On day 11 of this experiment, however, the Pc group had a lower parasitaemia than the PcV group ($p<0.05$ at 95% CI) (see Appendix 2 for individual experiments). Again when the two experiments were combined no significant difference was seen between the two groups at any time point (Figure 3.4).

An important observation made here was that parasitaemia levels were seen to be very high in some animals of both groups (Pc and PcV), up to 60% in some animals. High parasitaemia levels on their own, however, carried no prognostic significance since some mice made a full recovery. What did seem to be important in determining prognosis was the degree of red cell fragmentation. Animals with great red cell fragmentation seemed to die while the others seemed to recover. If this is true for humans as well, then it could perhaps be used to determine which patients to treat with a blood transfusion.

3.3. Effects of NISV on the clinical status of mice

3.3.i. Mortality

In the first experiment at Strathclyde University (S.1), the Pc group had 40% mortality, while the PcV group had a mortality rate of 20%. In the subsequent experiments, however, rates were seen to be higher (although not significantly) in the infected, NISV-treated group (PcV), even in those experiments showing a less severe disease in the treated group (Figure 3.5). Furthermore, in one experiment conducted at Strathclyde University, 20 % of animals were found dead even before being infected but after treatment with NISV. There were no signs of injury in these animals to attribute their death to fighting and to date it is not really known what caused their death. This particular experiment was terminated in case the mice were infected with a pathogen present in the animal house.

Among the mice that died some were found to be very warm as if death had occurred recently. Upon dissection, it was noted that the majority of these mice had very distended bladders (3-6ml) indicating urinary retention for some time prior to death.

3.3. ii. HHSS (Hassan Health Status Score):

Apart from mortality rates, a health status score was developed to allow a quantitative comparison of the animals' health in the two groups. It was based on a table used for mice infected with *Toxoplasma gondii* in which disease is divided into severe, moderate and mild (Animal House, Strathclyde University). More features were added to this table and a score was given to each feature (see Material and Methods). Initially bowel motion and urine appearance were included to describe diarrhoea, and haematuria. Subsequently they had to be excluded as these two features were not always observed during the period when attending the animals.

The results shown here represent a combination of two experiments conducted at each venue. In the first two experiments (S.1 and S.2), the PcV group had a higher score than the Pc group on days 11 and 12 post-infection, while in the second set of experiments conducted at Glasgow University (G.1 and G.2), the Pc group had a higher score than the PcV group between days 12-14 post-infection ($p<0.05$ at 95% CI) (Figure 3.6.a and b).

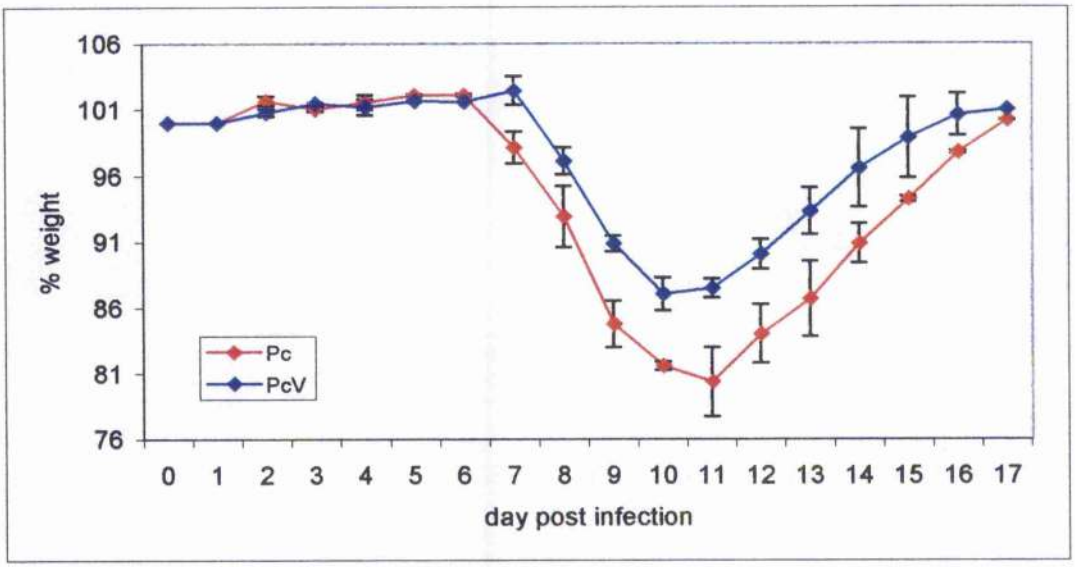


Figure 3.1. Percentage weight of 10-12 week old male BALB/c mice infected with *P. chabaudi* AS. Pc; animals infected with $1x10^5$ pRBC on day 0 and received 100µl PBS s.c. on days -28 and -14. PcV: animals infected with 10^5 pRBC on day 0 and received 100µl NISV s.c. on days -28 and -14. Results represent combined data of two experiments conducted at the same venue (Strathclyde University) containing 10 and 20 animals per group in experiments S.1 and S.2 respectively. The points represent the combined means and the bars are the standard errors of the means.

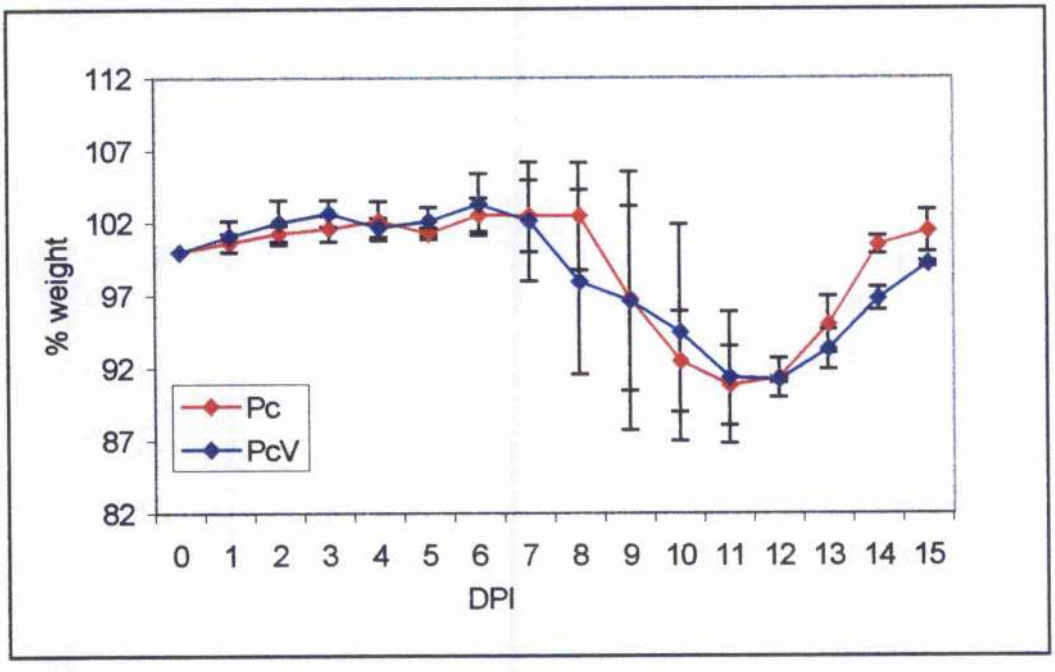


Figure 3.2. Percent weight of 10-12 week old male BALB/c mice infected with *P. c* AS. Pc and PcV are groups infected with $1x10^5$ pRBC on day 0 and injected subcutaneously with 100µl of PBS or NISV respectively on days -28 and -14. Results represent means of combined data of two experiments conducted at the same venue (Glasgow University) and error bars are the standard errors of the means.

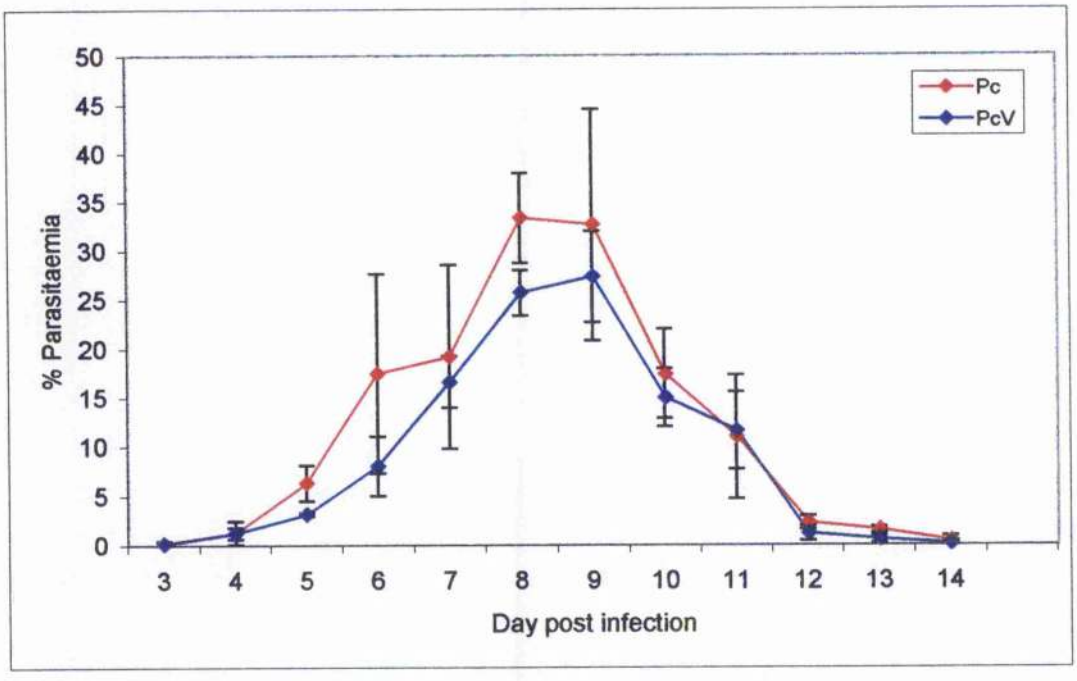


Figure 3.3. Percent parasitaemia in 10-12 week old male BALB/c mice infected with *P. c.* AS. Pc, PcV; animals infected with $1x10^5$ pRBC on day 0 and received subcutaneous injection of 100µl PBS and NISV respectively on days -28 and -14. Results represent combined data of 2 experiments conducted at the same venue (Strathclyde University) containing 10 and 20 animals per group in experiments S.1 and S.2 respectively. The points represent the combined means of the two experiments and the bars are the standard errors of the means.

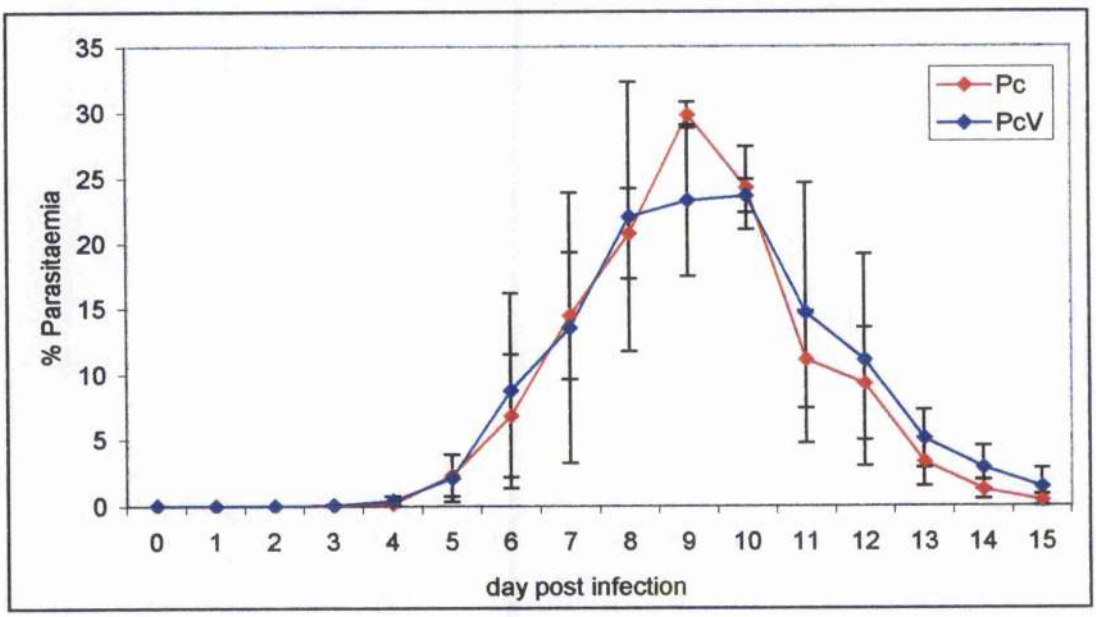
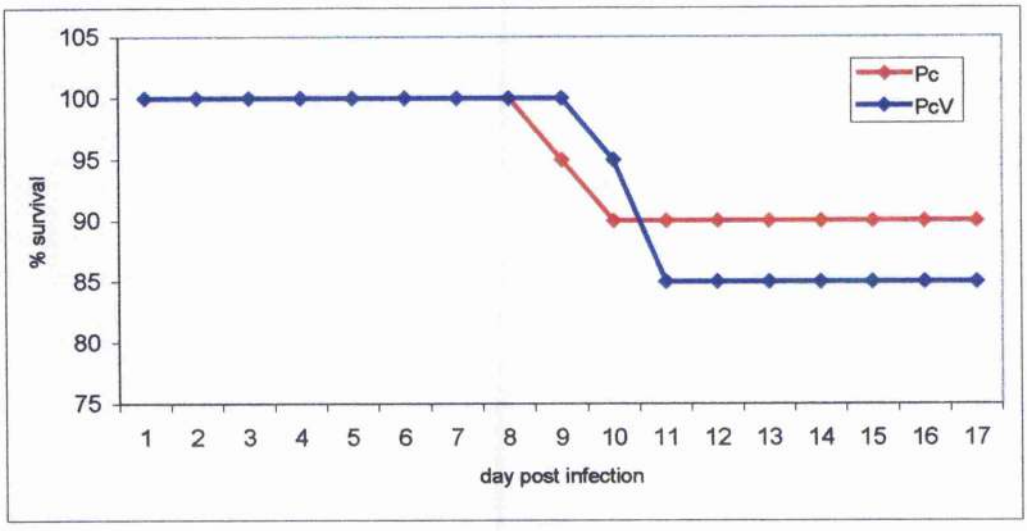
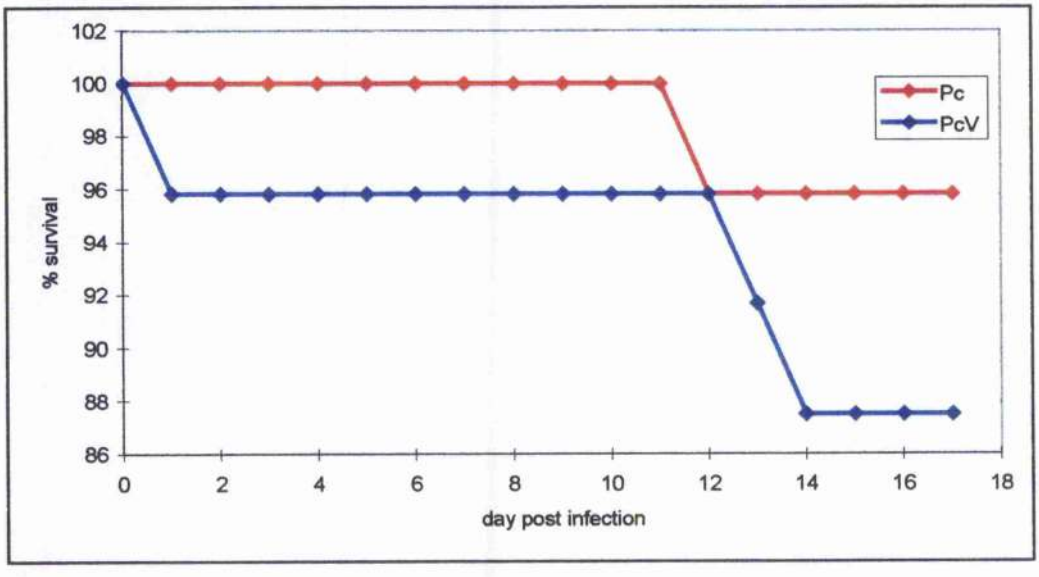


Figure 3.4. Percent parasitaemia of 10-12 week male BALB/c mice infected with *P. c.* AS. Pc and PcV groups were infected with $1x10^5$ pRBC on day 0 and injected subcutaneously with 100µl of PBS or NISV respectively on days -28 and -14. Results represent means of combined data of two experiments G.1 and G.2 conducted at same venue (Glasgow University) and error bars are the standard errors of mean.

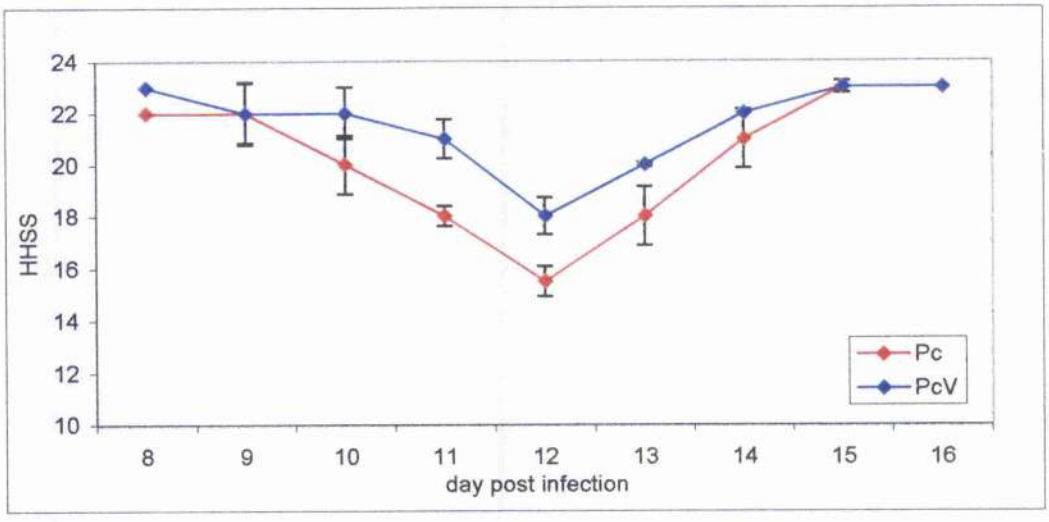


a

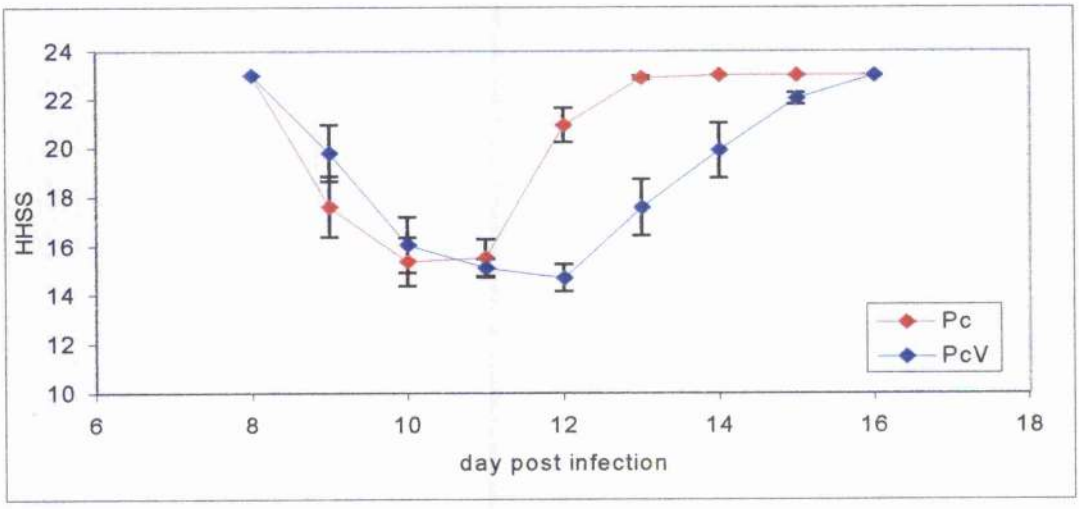


b

Figure 3.5.a, b: Percent survival of 10-12 week old male BALB/c mice infected with *P. c.* AS. 3.5.a. represents data from experiments S.2), while 3.5.b represents data from G.2. Pc and PcV are groups of mice infected with $1x10^5$ parasitized red blood cells on day 0 and received 100µl of PBS or NISV respectively on days –28 and –14.



a



b

Figure 3.6 a, b: Health status score of male 10-12 week old BALB/c mice infected with *P. c.* AS. Pc and PcV are groups of mice infected with $1x10^5$ pRBC on day 0 and injected subcutaneously with 100µl PBS or NISV respectively on days -28 and -14. 3.6.a; data from experiment S.2 and 3.6.b; data from experiment G.2

Discussion:

It can be seen from the experiments described in this chapter that conflicting results with regards to the animals weights, level of parasitaemia and health status score were obtained at the two venues (Strathclyde and Glasgow Universities). If anything then these experiments show the importance of repeating experimental work to confirm or refute any conclusions drawn from the initial results.

In these experiments, animals weight is expressed as a percentage of their weights on day 0. A better method of expressing weight loss, however, would have been the method of pair feeding which involves measuring the amount of food consumed by the ill animals and providing this amount of food to the control animals i.e. to equalize the amount of food consumed by both groups. This method would clearly show that despite consuming the same amount of food, the infected animals were indeed losing more weight indicating that the weight loss in them was not only a function of loss of appetite but also the catabolic process during their illness. It has to be emphasized, however, that the weight measurements in the ill animals is an under estimate of their catabolism since some of this weight is a measure of the enlarged spleen and to a lesser extent the liver.

Several possibilities which could explain the contradictory results were suggested. Initially it was thought that the NISV used in the third experiment (G.1) were not made properly and so did not produce their desired effects. This possibility, however, was overcome by obtaining the NISV fresh from the makers themselves (Brewer *et al.*, Division of Immunity, Infection and Inflammation, Glasgow) who were confident about the NISV although, they no longer checked their morphology under the microscope routinely.

Another possible explanation for the variation of the results was the possible infection of some animals with murine hepatitis virus (MHV) giving rise to variable results. This possibility too was rejected because the animals used were old enough to have cleared such an infection that is thought to affect younger mice. Another possible explanation

was that perhaps animals at the first venue had co-infections with pinworm, which could have affected the outcome of their illness, while animals at the second venue were claimed to be free of any such infestations. This was later discarded as some mice from the first venue were dissected and examined for worms and were found to be clean. This, however, does not exclude infestation by other organisms.

It was also thought that perhaps the amount of PABA taken in the diet by the animals at the two venues was different. As mentioned earlier, PABA was shown to have an effect on the susceptibility of mice to malaria infections (Maegraith, Deegan and Sherwood Jones, 1952). Both venues, however, used tap water from the same main city supplies without adding any PABA. Also both venues used the same pellet diet CRM(p) obtained from the same supplier. This, however, does not eliminate the possibility of the water pipes in either venue being colonized by some pathogens causing the above variation in the outcome of the illness.

Difference in the virulence of the parasites used in the different experiments was also thought of as a possible explanation for the contradictory results but all parasites were obtained from the same parasite parents kept in the same place at University of Glasgow.

An important point to note is that some mice in the first venue (Strathclyde University) exhibited clinical features, which were not seen at Glasgow University. Among these were gross oedema affecting one mouse in the infected, untreated (Pc) group which also showed lipids in its plasma suggesting a clinical picture of nephrotic syndrome. In humans, nephrotic syndrome (hypertension, oedema, hyperlipidaemia and proteinuria) is reported mainly with *P. malariae* (Souhami and Moxham, 1998) malaria rather than *P. falciparum* while to my knowledge no such condition had been reported in *P. chabaudi chabaudi* AS in mice. Another feature was the development of coprophagia in one mouse during the recovery phase and lasted for many days until the mouse eventually died possibly due to malnutrition since it refused to eat its normal diet. One last strange feature exhibited in one mouse was the running around in circles, which also lasted for several days. It is difficult to say whether it had developed a peripheral or

central neurological problem since none of the mice was studied by post-mortem. Whether these features were due to *P. c.* AS or due to some other infection occurring at this venue is not known. It is also worth noting that these features were seen later in the infection, during and after apparent clinical recovery.

In view of the above results, it becomes evident that the difference in the outcome of the experiments was venue-dependent. This, however, is not entirely true since retrospective examination of the results of more experiments revealed a wider variability in the results which were independent of the venue. In one of the experiments conducted at Strathclyde University, four mice were found dead shortly after the administration of the second dose of the NISV and before infection. The animals did not show any signs of injury to attribute their death to fighting. This experiment was, therefore, terminated in case the animals had been infected with another pathogen in the animals house. Also, the initial experiment conducted by the manufacturers of the NISV (Brewer, Strathclyde University) showing a reduced weight loss in the *T. gondii*-infected mice and treated with empty NISV does not make any reference to the presence of oedema which could give false positive results with regards to the animals' weight, nor does it make reference to other clinical parameters to assess the animals (Brewer, 1993). Moreover, in experiment S.1 effects of pre-infection treatment with NISV was investigated not only on *P. c.* AS-infected mice but also on *T. gondii*-infected mice using the same batch of the NISV. As mentioned earlier, in this experiment, the NISV-treated, *P. c.* AS infected group developed a less severe disease than the untreated group. However there was no difference between the NISV-treated and untreated, *T. gondii*-infected animals.

The difference between the two groups in any experiment could be a mere chance since it was also noticed between animals of the same group but in different cages. Also, although the difference in parasitaemia or weight loss between the two groups (Pc and PcV) was significant at some time points ($p<0.05$ at 95% CI), this by no means eliminates false positive results due to chance. This is because p value of <0.05 at 95% CI simply means that the difference between the groups has less than 5% probability of being due to chance. Moreover, there was always a mouse which was much worse than

others of the same group and a mouse which seemed to be hardly affected. In addition, one mouse at Strathclyde University had malformed incisors, while at Glasgow University one of the animals had situs invertus. Both these animals were eliminated from the experimental work. It is obviously possible that the differences seen in weight loss and percent parasitaemia could be due to errors made by the observer but this does not explain the differences seen in the HHSS for which a bystander from the animal house was used as a witness (Jack Keys and David McGlaughlan at the first and second venue respectively). Another point indicating that the difference in the outcome between the NISV-treated and untreated, was due to chance is the fact the difference between the two groups occurred at different time intervals in the different experiments.

This significance of urinary retention is not known but it could indicate the death of these animals from exhaustion due to increased sympathetic drive trying to put up with the stress of the disease. The red cell fragmentation observed in mice dying soon afterwards is worth investigating in human malaria. This could be useful as a prognostic sign and in determining treatment by blood transfusion.

From the above results, it becomes evident that the difference between the NISV-treated and untreated male BALB/c mice infected with *P. c.* AS was not due to the use of NISV. Indeed when the four experiments are combined together, no significant difference between the two groups with regards to degree of weight loss or level of parasitaemia is seen at any time point (Figures 3.7, 3.8).

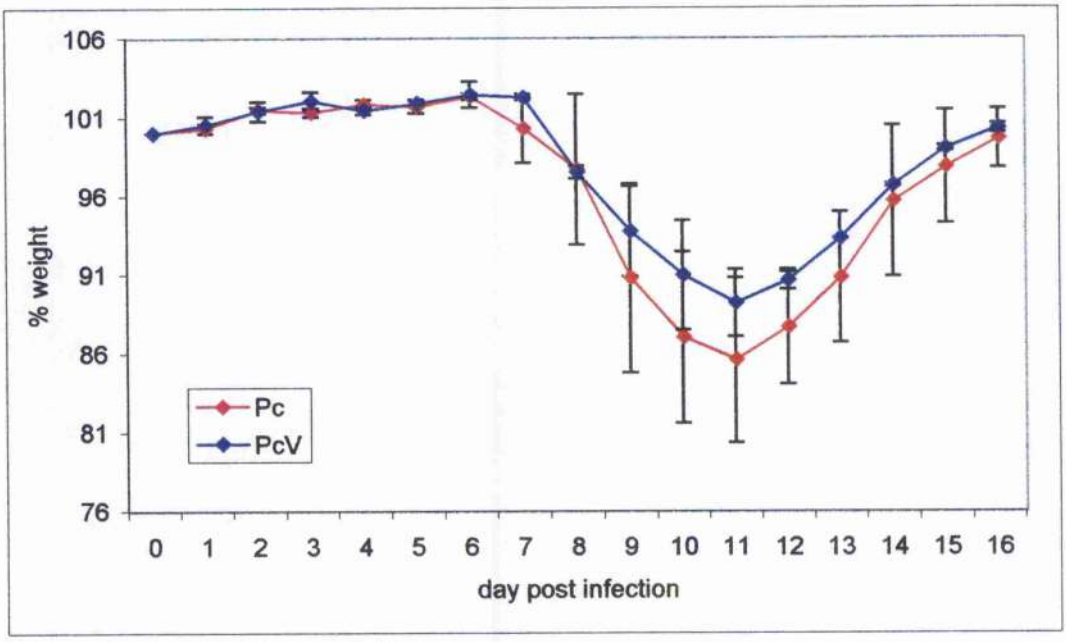


Figure 3.7: Percent weight of male BALB/c mice infected with *P. c.* AS. Results represent combined data obtained from 4 experiments. Pc and PcV are 2 groups infected with $1x10^5$ pRBC on day 0 received 100µl PBS or NISV respectively on days -28 and -14. Although the NISV treated group shows less weight loss than the untreated group, this however is not significant at any time point

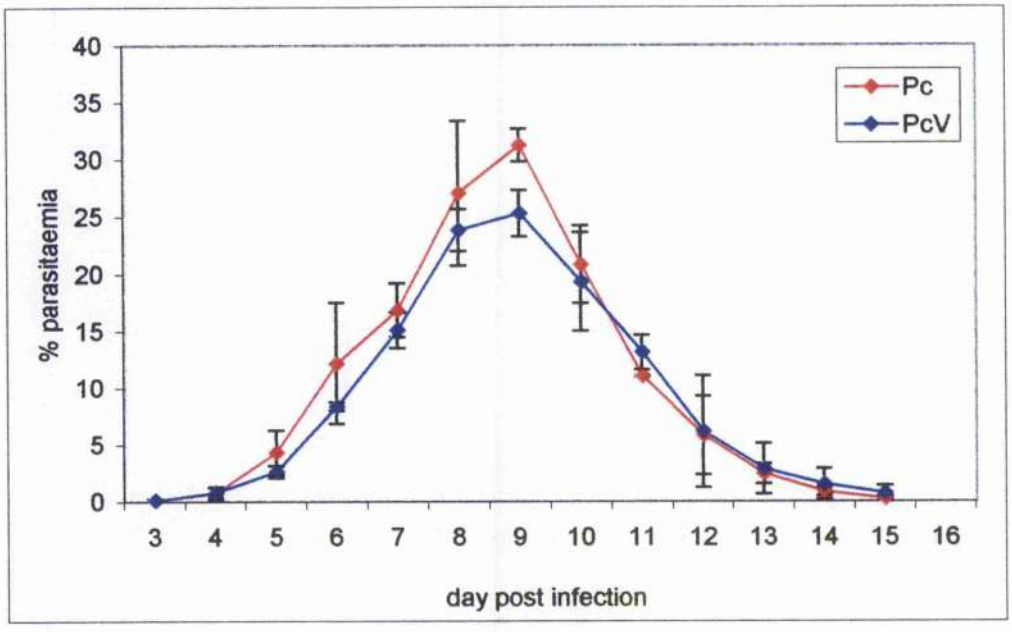


Figure 3.8: Percent parasitaemia in 10-12 week old male BALB/c mice infected with *P. chabaudi* AS. Results represent combined data obtained from 4 experiments. Pc and PcV were injected with $1x10^5$ pRBC on day 0 and received 100µl of PBS and NISV respectively on days -28 and -14.

Chapter 4

Effects of pre-infection administration of NISV on TNFα levels in male BALB/c mice infected with *P. chabaudi chabaudi* AS.

Introduction

As mentioned in the previous chapter, pre-infection treatment of *P. c.* AS-infected mice with NISV led to a lower level of parasitaemia, a less degree of weight loss and a higher health status score than the untreated mice in the initial experiments (S1 and S2). Since high levels of TNFα have been reported to cause cachexia during infections (Beutler *et al.*, 1985), it was decided to measure the TNFα levels in NISV-treated, *P. c* AS-infected mice and compare them with those in the untreated, infected mice.

Tumor necrosis factor:

Tumor necrosis factor alpha (TNFα) is a monokine that was discovered by two different groups of researchers and accordingly was given two different names. It was initially identified in the serum of mice, primed with BCG and injected with LPS two hours previously, as a factor capable of causing necrosis of some subcutaneous tumors in mice (Carswell *et al.*, 1975). It was also discovered and purified by Beutler and colleagues (1985) investigating weight loss during infection of animals with trypanosomes and was, therefore, named cachectin. It was not long before Beutler and colleagues noticed that the amino terminal sequence of the mouse cachectin and the human TNF shared a strong homology suggesting that they were in fact the same molecule (Beutler *et al.*, 1985). When the TNF cDNA was cloned, its sequence exactly predicted the amino terminal sequence of the mouse cachectin (Fransen *et al.*, 1985, Caput *et al.*, 1986). Immunogenic studies and bioactivity measurements of the two molecules confirmed that the two molecules were the same (Beutler and Cerami, 1988).

TNFα is encoded by a gene located on chromosome 17 in mice, close to the MHC II region (Müller *et al.*, 1987, Nedospasov *et al.*, 1986), and on the short arm of chromosome 6 in humans (Nedwin *et al.*, 1985) closely linked to HLA B region (Carroll *et al.*, 1987). It is not known whether these locations have any significance.

TNFα is initially synthesized as a non-glycosylated transmembrane protein of approximately 26 kD. A 17 kD fragment is proteolytically cleaved off the plasma membrane. This released fragment aggregates *in vitro* to produce dimers, trimers and pentamers but the bioactive form is thought to be a 52kDa homotrimer in the shape of a pyramid (Eck and Sprang, 1989, Jones *et al.*, 1989). Three receptor-binding sites are at the base of this pyramid (Van Ostade *et al.*, 1991) allowing simultaneous binding to more than one receptor. In addition to the 17kD form, a 26kD membrane-bound form of TNFα that was detected by anti-TNFα polyclonal antibodies also exists on activated macrophages monocytes (Kriegler *et al.*, 1988). The authors suggested that this form is rapidly induced on monocytes at sites of inflammation. In fact, membrane-bound form of TNFα had been identified before by Decker and colleagues. They had reported TNFα-dependent lysis of tumor cells by activated macrophages that had been fixed by paraformaldehyde before the addition of the target cells and in the absence of any measurable secreted TNFα. Indirect radioimmunassay studies confirmed the presence of TNFα on the surface of activated macrophages. The authors suggested that the lytic activity exhibited by these macrophages occurred through direct contact with the target cells (Decker *et al.*, 1987).

TNFα is mainly produced by mononuclear phagocytic cells (Old *et al.*, 1985). In C57Bl/6 mice it was found to be produced by all macrophages tested including alveolar, hepatic, peritoneal, bone marrow-derived macrophages and peripheral blood monocytes (Decker *et al.*, 1987). Several other cell types have been reported to produce TNFα. Among these are T lymphocytes which produce TNFα in response to phorbol myristate acetate and calcium ionophore (Cuturi *et al.*, 1987) and NK cell produce TNFα in small quantities (Degliantoni *et al.*, 1985). Mast cells have been found to contain TNFα stored in their granules (Gordon and Galli, 1990). Human B lymphocytes transformed by Epstein Barr virus have also been reported to secrete TNFα (Williamson *et al.*, 1983).

Apart from LPS, which is the most potent stimulator of TNFα production, several factors have been shown to induce the production of TNFα. Certain viruses, trypanosomal and plasmodial lysates and certain Gram positive bacteria like staphylococci have been reported to induce TNFα production *in vitro* although in

relatively small quantities (Aderka *et al.*, 1986). IFNγ, although not capable of inducing TNFα production on its own, increases the rate of TNFα gene transcription when co-administered with LPS (Beutler *et al.*, 1986) and upregulates the expression of TNFα receptors on the target cell (Tsujimoto *et al.*, 1986). IL-2 is also thought to increase the rate of TNFα gene transcription (Nedwin *et al.*, 1985). Malaria exoantigens have been reported to stimulate the production of TNFα from macrophages and their active moiety has been found to be phosphotidylinositol and inositol monophosphate since antibodies to these specifically inhibit TNFα induction by malaria exoantigens (Bate *et al.*, 1992). Glucocorticoids, on the other, hand suppress TNFα biosynthesis *in vitro* if administered to macrophages prior to LPS. Indeed pre-LPS glucocorticoid administration to animals protects them from a lethal dose of LPS (Beutler *et al.*, 1986). Cyclosporin A was reported to inhibit TNFα bioactivity but without inhibiting the expression of TNFα mRNA (Nguyen *et al.*, 1990).

TNFα mRNA concentrations rise within minutes in macrophages exposed to LPS *in vitro* and detectable amounts of TNFα are secreted within 20-30 minutes (Beutler and Cerami. 1988). TNFα production, however, is inhibited by addition of actinomycin D indicating that messenger of TNFα is rapidly induced by LPS (Gifford *et al.*, 1984). The levels of mRNA reach their peak 2-3 hr after induction, declining slowly thereafter to approach levels seen in non-stimulated cells after 15-20 hr despite the continual presence of the stimulus (Beutler and Cerami, 1988). TNFα production ceases within 4-6 hr following contact with LPS. In this time period during which TNFα is produced, continual exposure to LPS is essential as removal of LPS led to cessation of TNFα production which was resumed when LPS was again added to the cultures (Gifford *et al.*, 1986).

In vivo studies have shown that after endotoxin injection into rabbits, TNFα appears in the circulation within 20 minutes (Beutler *et al.*, 1985) and reaches peak levels between 90 min and 2 hours, declining thereafter to reach basal levels within 5-6 hr. In mice, radio-iodinated TNFα was found to have a plasma half-life of 6-7 minutes (Beuter *et al.*, 1985).

Two discrete types of TNFα receptors of 55 and 75 kDa and of different affinities have been identified at the cell surface of a variety of cell lines including human peripheral blood monocytes (Hohman *et al.*, 1989, Brockhause *et al.*, 1990). There are two receptors in mouse too (Lewis *et al.*, 1991). The murine TNF-R1 and TNF-R2 have 64% and 62% homology to human TNF-R1 and TNF-R2 respectively (Lewis *et al.*, 1991, Goodwin *et al.*, 1991). It has been reported that high affinity receptors are found not only on cells sensitive to TNFα cytotoxicity such as L929 but also on cell types resistant to TNFα cytotoxicity such as FS-4 (Tsujimoto *et al.*, 1985). The majority of cell types seem to express both types of the receptors known as p55 and p75 (TNF-R1 and TNF-R2 respectively). There is no homology between the TNF-R1 and TNF-R2 in their intracellular portions indicating that the intracellular signaling pathways are different (Lewis *et al.*, 1991). The extracellular portions of both TNF-R1 and TNF-R2 are shed and retain their ability to bind TNFα (Engelman *et al.*, 1989). The majority of the TNF-mediated biological activities such as cell death, gene induction and cytokine production have been attributed to TNF-R1 (Lewis *et al.*, 1991). TNF-R2 is thought to be involved in T cell development and proliferation of cytotoxic T cells (Tartaglia *et al.*, 1991, Tartaglia *et al.*, 1993). TNF-R2 KO mice, however, have been reported to show a normal T cell development and activity, an increased resistance to TNF-induced death and a decrease in tissue necrosis indicating that this receptor plays a role in the necrotic effects of TNFα (Erikson *et al.*, 1994). It has been shown that following binding of TNFα to p55, the complex becomes internalized and eventually degraded in lysosomes (Mosslemans *et al.*, 1988, Imamura *et al.*, 1987).

TNFα has been found to exert multiple effects on a variety of cell types. It has been shown to have many metabolic effects including the inhibition of lipoprotein lipase on fat cells (Beutler *et al.*, 1985) which causes break down of adipose tissue and hypertriglyceridaemia. It also suppresses the differentiation of adipocytes (Torti *et al.*, 1985). Moreover it antagonises the lipogenic effects of insulin (Pekala *et al.*, 1983). Together these effects inhibit storage of fat, leading to wasting.

TNFα has powerful effects on the activation and differentiation of monocytes resulting in further release of TNFα (Hohmann *et al.*, 1990). Upon activation of

macrophages there is the stimulation of a pathway leading to the production of NO, which has marked parasiticidal activities (Ding *et al.*, 1986, Nüssler *et al.*, 1992). TNFα also stimulates the production of IL-6 and PgE2 by macrophages and together they induce complex intracellular mechanisms, which control the transcription and translation of TNFα mRNA (Han, Heuz and Beutler, 1991).

TNFα is chemotactic for neutrophils *in vitro* and induces the expression of ELAM-1 on endothelial cells, which is a potent inducer of neutrophil migration. TNFα, along with interleukin-1, has also been shown to enhance neutrophil adhesion to endothelium *in vitro* (Pohlman *et al.*, 1986, Gamble *et al.*, 1985). It augments their phagocytic activity and increases their cytotoxicity to certain microorganisms (Shalaby *et al.*, 1985). TNFα itself has been found to be a weak stimulant of neutrophil respiratory burst (Klebanoff *et al.*, 1986). On the other hand it also induces the production of IL-6 and IL-8 by endothelial cells. Although these cytokines are chemotactic, they have an inhibitory effect on leukocyte adhesion (Gimbrone *et al.*, 1989).

Recombinant human TNFα was found to increase TNFα receptors on IL-2-stimulated T cells. TNFα also promotes the expression of IL-2 receptor and HLA-DR antigen on lymphocytes and augments the IL-2-dependent IFNγ production by the T cells (Scheurich *et al.*, 1987)

TNFα is an endogenous pyrogen, causing fever both directly by acting on the hypothalamus and indirectly by triggering peripheral production of IL-1. Injection of rabbits with human rTNFα causes a brisk monophasic fever (Dinarello *et al.*, 1986).

In the case of malaria, it was initially shown that tumor necrosis serum (serum obtained from BCG-infected mice, two hr following an intravenous injection of LPS) was toxic to blood stages of *P. falciparum* (Haidaris *et al.*, 1983) *P. yoelii* and *P. berghei* (Taverne *et al.*, 1987) *in vitro*. Rabbit antiserum against partially purified TNF protected the intra-erythrocytic forms of *P. falciparum* from the effects of the BCG-LPS serum (Haidaris *et al.*, 1983). By using rTNFα, however, it was shown that TNFα itself did not have any toxic effects on the *P. falciparum in vitro* (Jensen, van

de Waa and Karadsheh 1987, Taverne *et al.*, 1987, Kumaratilake, Ferrante and Rzepczyk, 1990) but it rather led to the production of intermediates, which could be the effecter molecules in killing the parasites. It was initially thought that the *in vitro* killing of the blood stage parasites by the tumour necrosis serum was due to reactive oxygen intermediates (ROI) since mitogenic stimulation of peritoneal macrophages 14 days following treatment with BCG gave an optimal hydrogen peroxide release (Nathan, 1997). Furthermore, hydrogen peroxide had been reported to be toxic to blood stage parasites *in vitro* (Dockrell *et al.*, 1983). Reactive oxygen intermediates, however, are short lived and their effects could be blocked by antioxidants, which is not the case for the parasiticidal activity of tumour necrosis serum. It was, therefore, thought that ROI could not be responsible for this toxicity (Geary *et al.*, 1986). Instead lipid peroxides, which are produced by reaction of plasma lipoproteins with ROI and which are unaffected by antioxidants (Morel *et al.*, 1984), were suggested to be responsible for the *in vitro* anti-parasitic activity with perhaps other factors (Rockett *et al.*, 1988). The authors suggested that if these oxidized lipids were taken up by the parasites, they could have a direct toxic effect on the parasite by cross linking their proteins or by destabilizing the erythrocyte membrane. Subsequent studies showed that serum of CBA/CaH mice undergoing a successful immune response against *P chabaudi* infection was not toxic to *P. falciparum* in culture unless the infection and the TNFα levels of the mice were high enough to cause illness. The authors, therefore, suggested that the downstream products of TNFα required activated macrophages to cause intra-erythrocytic death of parasites (Clark, Cowden and Butcher, 1990). A synthetic peptide corresponding to residues 70-80 of the TNFα molecule ($TNF\alpha_{70-80}$) was reported to enhance human polymorphonuclear killing of *P. falciparum in vitro* and reduce *P. chabaudi* parasitaemia in mice (Kumaratilake *et al.*, 1990, 1995). The $TNF\alpha_{70-80}$ peptide did not increase the expression of adhesion molecules on endothelial cells and inhibited the TNFα-induced increase in *P. falciparum*-infected erythrocytes binding to endothelial cells. The authors suggested that the protective effects of TNFα could be retained while toxic effects are eliminated by using selected characterized subunits of TNFα (Kumaratilake *et al.*, 1995). Furthermore, a recent report has shown that addition of TNFα to cultures of human monocytes and *P. falciparum* infected erythrocytes increased the phagocytic

index of the monocytes from 3.8-7.8 in the presence of serum containing *P. falciparum* antibody (Muniz-Junqueira *et al.*, 2001).

In vivo studies revealed both a protective and a harmful effect for TNFα in malaria. A protective role for TNFα was evident in male CBA/CaH mice infected with *P. chabaudi adami* and treated with human rTNFα via an osmotic pump. These animals developed a lower parasitaemia, earlier crisis forms and an earlier decline in parasitaemia than the untreated group (Clark *et al.*, 1987). Treatment with 10^3-10^5 units of human rTNFα also resulted in 80% survival and significant decrease in the peak parasitaemia in susceptible A/J mice infected with *P. c.* AS. Moreover, all the surviving mice recovered by week 4 and were completely protected against a challenge infection. An important finding in these mice was that a higher TNFα dose was needed to produce protection as the infective dose of pRBC increased. Giving the same treatment to resistant C57Bl/6 mice, on the other hand, did not produce any effect except that a dose of TNFα higher than 10^5 U resulted in 100 % mortality (Stevenson and Ghadirian, 1989). This indicated that the resistant mice did produce enough TNFα to protect themselves and exceeding a certain levels of TNFα resulted in harmful effects. Furthermore, treatment of *P. berghei* K173-infected female C57Bl/6 mice prevented the development of experimental cerebral malaria (ECM) and also reduced parasitaemia in those mice that did not develop ECM but not in those that did developed it (Postma *et al.*, 1999). Repeated injection of murine rTNFα into *P. yoelii* 17X-infected out bred mice reduced their parasitaemia and significantly prolonged their survival (Taverne *et al.*, 1987). Transgenic mice carrying a modified human TNFα β-globin chain and infected with *P. yoelii* had a 10 fold lower parasitaemia at its peak than the wild type mice and those infected with *P. chabaudi* had even a lower level of parasitaemia than their wild type counterparts. The decrease in parasitaemia was thought to be due to enhancement of macrophage activity against parasitized red blood cells by the human TNFα (Taverne *et al.*, 1994).

Recent work on TNFα receptor double knock out (p55, p75) resistant B6 x 129 mice did not, however, lead to any alterations in the parasite levels or in their clearance. The authors suggested that TNFα therefore was not critical for resolving blood stage infection with *P. chabaudi* AS malaria and its absence did not affect the early IL-12

production and Th1 response (Sam *et al.*, 1999). Other studies, on the other hand, have reported that although TNFα R1 may not be essential for the control of primary parasitaemia re-infection in such animals was as severe as the primary infection indicating that TNF-R1 was necessary for the development of memory responses (Li and Langhorne, 2000).

Other studies showed that both the timing and site of TNFα generation during infection were important. TNFα mRNA in the liver and spleen was found to be high early during infection in resistant C57Bl/6 mice infected with *P. chabaudi* AS, while high levels were found in the liver of susceptible A/J mice late during infection in whom circulating TNFα was also high during this period (Jacobs *et al.*, 1996).

On the other hand several studies indicated a role for TNFα in malaria pathology. Serum TNFα levels were found to be significantly higher in *P. berghei* ANKA-infected mice that developed cerebral malaria (CM), than those that did not develop it (Grau *et al.*, 1987c). In the animals that developed CM, local TNFα was also found by immunohistochemical staining, while it was absent in the animals that did not develop CM. Indeed treating the infected animals with a neutralizing anti-TNFα antibody before the appearance of CM dropped CM from 80-90% to 8.3%, but it did not protect them from death which was due to severe anaemia (Grau *et al.*, 1989). In addition, *P. berghei* K173-infected C57Bl/6J mice exhibited increased sensitivity to TNFα and doses as low as 2.5μg on days 8-10 were lethal. Depletion of macrophages which are the usual source of TNFα also prevented the development of cerebral malaria (Curfs *et al.*, 1993). Treating these mice with sublethal doses of TNFα (1.0ug/ml), however prevented the development of CM indicating dose-dependent effects of TNFα. Also, transgenic mice expressing high levels of TNFα receptor 1 fusion protein were markedly protected from death by cerebral malaria after infection with *P. berghei* (Garcia *et al.*, 1995).

Furthermore, injection of rTNFa into naïve mice induced systemic changes found in malaria such as hepatic midzone necrosis, hypoglycaemia and neutrophil adhesion to pulmonary blood vessels. No cerebral changes were seen, however, and the blood brain barrier which is interrupted in CM remained intact. The authors suggested that

the changes seen in CM in mice infected with *P.berghei* ANKA could in fact be due to locally produced TNFα from monocytes sequestered in the cerebral circulation rather than due to systemic TNFα levels (Clark *et al.*, 1990). Indeed work on murine cerebral malaria showed that TNFα is locally produced by microglia and astrocytes few days before the appearance of clinical features (Jennings *et al.*, 1998).

TNFα is thought to play at least a part in the murine malaria associated-anaemia. Treatment of *P. berghei* female BALB/c mice with rhuTNFα via an implanted osmotic pump led to reduction of pluripotent stem cells and erythroid progenitors in the bone marrow, splenomegaly and reduced incorporation of iron into erythrocytes. Treatment of these mice with anti TNFα serum lead to partial recovery of the bone marrow with an increase in the stem cells and erythroid progenitors plus increased incorporation of iron (Miller *et al.*, 1989). The role of TNFα in malaria-associated anaemia was also seen in transgenic mice for the human TNFα-β chain which excrete human TNFα. These mice were shown to develop anaemia thought by the authors to be due to increased erythrophagocytosis (Taverne *et al.*, 1994).

Circulating TNFα levels were also seen to correlate with CM in humans. In a study conducted on Gambian children it was shown that TNFα levels was twice as high in those who developed CM and 10 times higher in fatal cases than those with uncomplicated malaria (Kwiatkowski *et al.*, 1990). Serum TNFα levels were shown to correlate positively with the severity and mortality rates of *P. falciparum* in children in Malawi (Grau *et al.*, 1989, Kern *et al.*, 1989). Here high circulating TNFα levels were associated with hypoglycaemia, hyperparasitamia and age under 3 years (Grau *et al.*, 1993). Also higher TNFα and IL-6 were found in the circulation of patients with CM or complicated malaria than in patients with uncomplicated malaria (Ringwald *et al.*, 1993), and levels of both cytokines were found to be useful markers for *P. falciparuml* severity (Kern *et al.*, 1989). In one study, depletion of TNFα with the use of anti-TNFα monoclonal antibodies in Gambian children with cerebral malaria did not alter mortality but instead increased the neurological sequelae in the survivors. This was explained by the possible extended retention of TNFα in the

circulation by the antibodies and therefore prolongation of its effects (van Hensbroek *et al.*, 1996).

As mentioned earlier, TNFα is an enodogenous pyrogen (Dinarello *et al.*, 1986). In malaria fever has been attributed to TNFα. Fever in Gambian children with uncomplicated malaria was associated with moderately elevated levels of circulating TNFα (Kwiatkowski, 1990). Indeed neutralizing anti- TNFα monoclonal antibodies have been shown to inhibit fever in Gambian children with cerebral malaria (Kwiatkowski *et al.*, 1993).

In view of the above studies, it becomes evident that TNFα has dual protective/pathological effects depending on its levels. Certain TNFα levels are required to produce protective effects, but beyond these levels the pathological effects appear.

Inbred male BALB/c mice were infected with *P. chabaudi* AS. A group of these animals was treated with NISV prior to infection. The aim was to test the hypothesis that NISV suppress TNFα production from the macrophages (Brewer and Alexander, personal communication). The animals were followed up clinically (chapter 3), and with regards to various cytokines, IFNγ (chapter5), IL-4 (chapter 6) and TNFα, the results of which are presented here.

Results

4.1. Effects of NISV on plasma TNFα levels of *P. c* AS -infected mice.

The results shown here are from three experiments, the first two (S.1 and S.2) conducted at Strathclyde University while the last one (G.1) was conducted at Glasgow University. Inbred male BALB/c mice were infected with $1x10^5$ pRBC of *P. chabaudi* AS (n = 10, 20 and 20 in experiments S.1, S.2 and G.1 respectively) on day 0. They had received 100µl subcutaneously of either PBS or NISV on days -28 and -14 and referred to as the Pc and PcV groups respectively. Age and sex-matched

animals were also injected with PBS and NISV but received no infection and were referred to as PBS and NISV. Animals were bled into heparinised capillary tubes and samples of the same group were pooled and stored at –20°C until analysis.

In experiment S.1, no TNFα was detected early during the infection. TNFα was only detected in the Pc group late in the infection, on days 37-42 (Figure 4.1). It was therefore decided to reduce the amount of TNFα used in the starting standard from 20ng/ml. This was done serially until some levels were detected in the samples against standards starting from 2ng/ml. In experiment S.2, higher levels of TNFα were seen in the Pc group than the PcV group on days 9, 12 and 13 post-infection. TNFα peaked twice in the Pc group, on days 9 and 12 while in the PcV group it peak once only on day 9 post-infection (Figure 4.2). In this particular experiment, the peak parasitaemia occurred on day 9 while animals weight reached its nadir on day 8 post-infection. In both S.1 and S.2 experiments, the PcV group exhibited a less severe disease than the Pc group in terms of percent parasitaemia, percent weight loss and health status score (see Appendix 2). In experiment G.1, TNFα also peaked twice in the Pc group on days 9 and 10 and was significantly higher than the PcV group which showed one peak only on day 9 post-infection (Figure 4.3). In contrast to the previous two experiments, however, the PcV group exhibited a more severe disease (higher parasitaemia, more weight loss and lower health status score) than the Pc group in this particular experiment (see Appendix 2). No TNFα was detected in groups PBS and NISV (Figure 4.3).

4.2. Effects of pretreatment with NISV on whole blood TNFα

Whole blood was collected in one experiment only (G.2). Twenty five inbred male BALB/c mice per group were infected with *P. c* AS and treated with NISV or PBS as described above. Blood was collected from two or five animals per group via cardiac puncture or via the tail vein respectively at selected time points judged by the clinical status of the animals. Samples from the same group were pooled and 100µl of ddH2O were added to each 1ml of blood in order to break any clots prior to freezing. Samples were then stored at –20°C until further analysis.

TNFα was detected in both groups from day 3 until day 11 post-infection. On day 12 TNFα was seen in the PcV group only, while on day 14 it was detected in the Pc group only (Figure 4.4). TNFα was slightly lower in the PcV group on days 3, 6, 7 and 8 and slightly higher on days 4, 10 and 11 than the Pc group. TNFα levels peaked to around 400U/ml on day 21 (following recovery from primary parasitaemia) in both Pc and PcV groups with slightly higher levels seen in the Pc group. TNFα was measured in the PBS and NISV groups at one time point only (day 21 post infection). No TNFα was detected in the PBS group, while low levels (30U/ml) were seen in the NISV group. Since this experiment was done once only, it cannot be compared with other results. In this particular experiment, the PcV group exhibited a worse disease (see Appendix 2).

4.3. Effects of pre-infection treatment with NISV on *ex-vivo* splenic levels of TNFα

Results shown here cover two experiments (G.1 and G.2). Two animals per group from groups of inbred male BALB/c mice infected in the above experiments were sacrificed at selected time points during infection judged by the appearance of clinical features (days 1, 4, 7, 10 and 16 and days 6, 8, 10, 11, 14, 17 and 21 post-infection in experiments G.1 and G.2 respectively). Spleens were removed aseptically and exposed to various stimulating or control agents including ConA, parasitized red blood cells, non-infected red cells plus complete RPMI. Splenocytes were incubated at 37°C in 5% CO_2 in air for various periods (4hr, 6hr, 18hr and 24hr) because of the difficulty in detecting TNFα. Supernatants were stored at –20°C until further analysis.

TNFα was detected in only one experiment (G.1) and only at 4 hr incubation while all other attempts of detecting it in the other experiment (G.2) and at other time points failed despite the use of the same antibodies (Figure 4.5). The PBS and NISV groups were found to produce TNFα in response to ConA and pE on day 10 post-infection, while low levels were detected in response to ConA on day 1. TNFα was detected in the two infected groups (Pc and PcV) on day 1 post-infection when it was more than double in the Pc group (25U/ml) than the PcV group (12U/ml) and on day 10 in

response to ConA, pE and nE, but with higher levels in the PcV than the Pc group (Figure 4.5). Some TNFα (6U/ml) was also detected in the PcV group on day 4 post-infection in response to nE. Peak parasitaemia occurred on days 8 and 9 in the PcV and Pc groups respectively. In this particular experiment, the PcV group developed a more severe disease than the Pc group in terms of percent parasitaemia, percent weight loss and health status score (see Appendix 2).

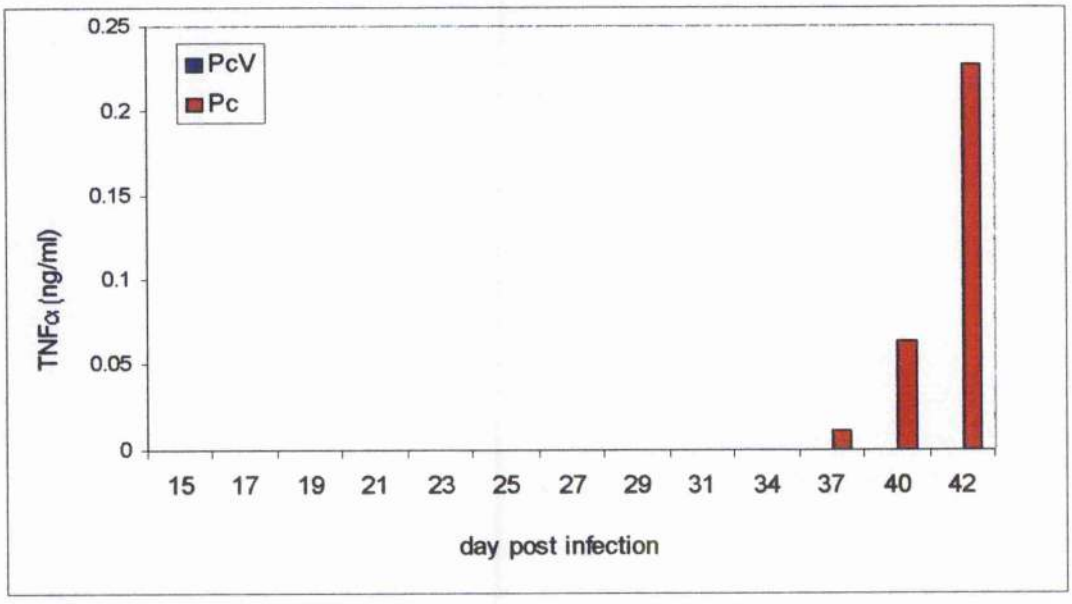


Figure 4.1 (experiment S.1): TNFα levels in the plasma of 10-12 week old male BALB/c mice infected with *P. c.* AS. Pc and PcV are mice infected with $1x10^5$ parasitised red blood cells on day 0 and received 100µl of PBS or NISV respectively on days –28 and –14. Results represent pooled samples of 5 animals per each time point.

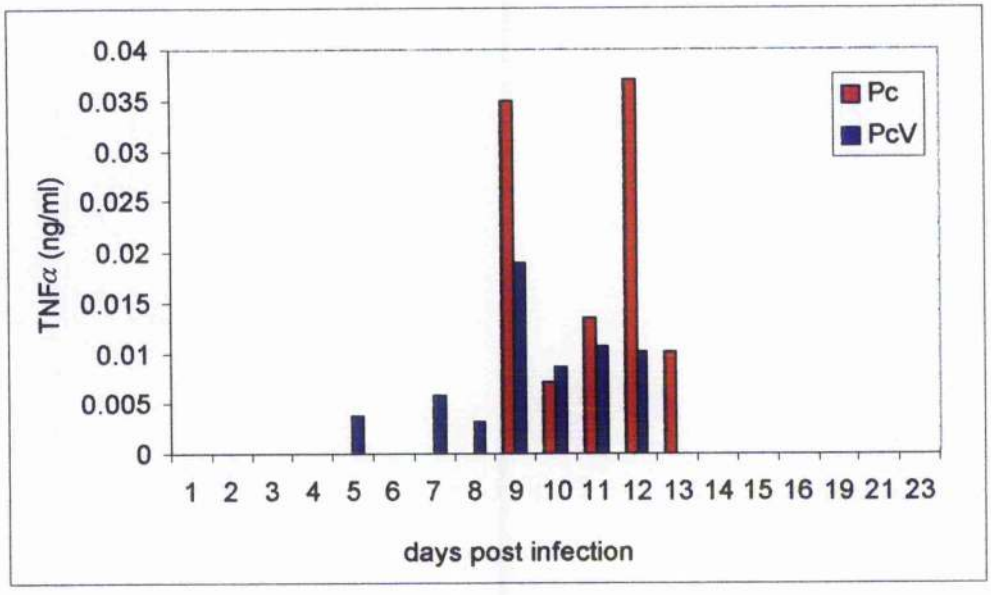


Figure 4.2 (experiment S.2): TNFα concentration in plasma samples of 10-12 week old male BALB/c mice per group infected with *P. chabaudi chabaudi* AS. Pc and PcV groups were injected intravenously with 1x 10^5 pRBC on day 0 and had received 100µl of subcutaneous PBS and NISV respectively on days -28 and -14. Results represent pooled samples of 5 animals per group at each time point.

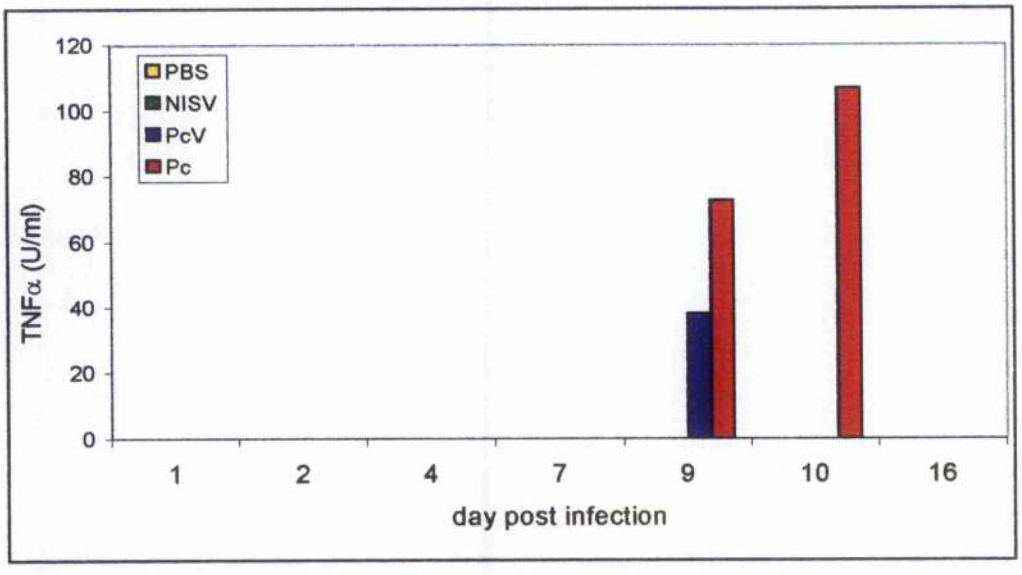


Figure 4.3: TNFα levels in the plasma samples of 10-12 weeks old male BALB/c mice infected with *P. c* AS. PBS and NISV groups contained 10 animals each and received subcutaneous 100µl PBS and NISV respectively on days -14 and -28. Pc and PcV each contained 20 animals, received 100µl subcutaneously of PBS and NISV respectively on days -28 and -14 and were injected i.p. with $1x10^5$ pRBC on day 0. Results represent pooled samples of 2 mice or 5 mice per group at each time point.

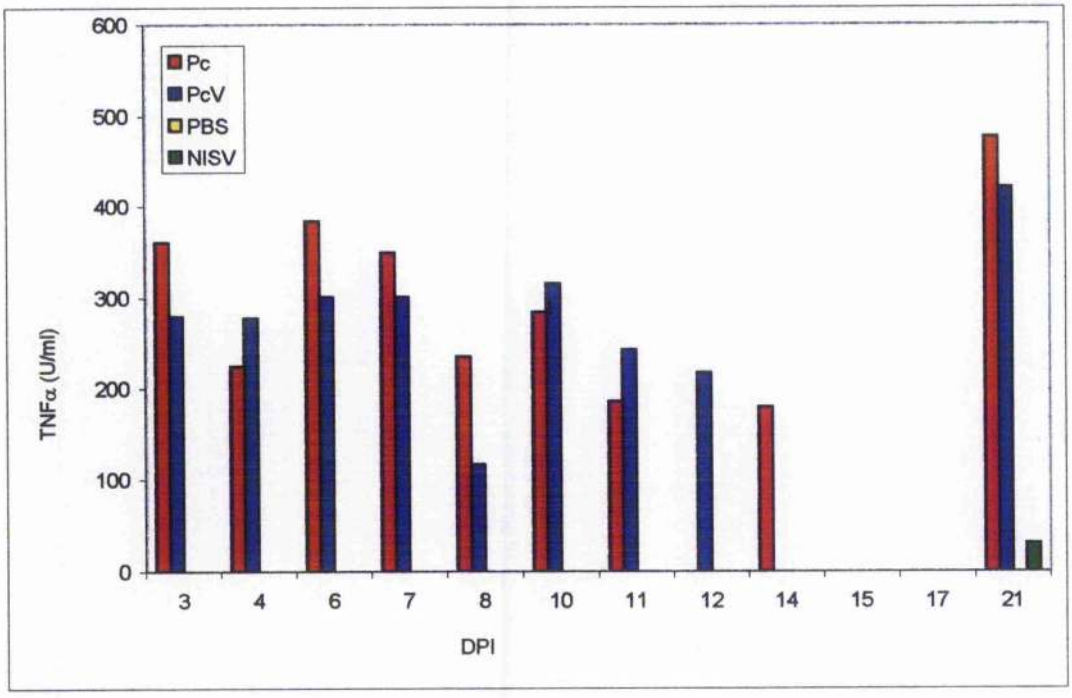


Figure 4.4: TNFα concentration in whole blood of 10-12 week old male BALB/c mice infected with *P. c* AS. Groups Pc and PcV were infected i.p. with $1x10^5$ pRBC on day 0 and had received 100µl subcutaneously of PBS or NISV respectively on days -28 and -14. PBS and NISV groups were similarly injected with 100µl of PBS or NISV respectively but without infection. Results represent pooled samples of 2 animals per group at each time point.

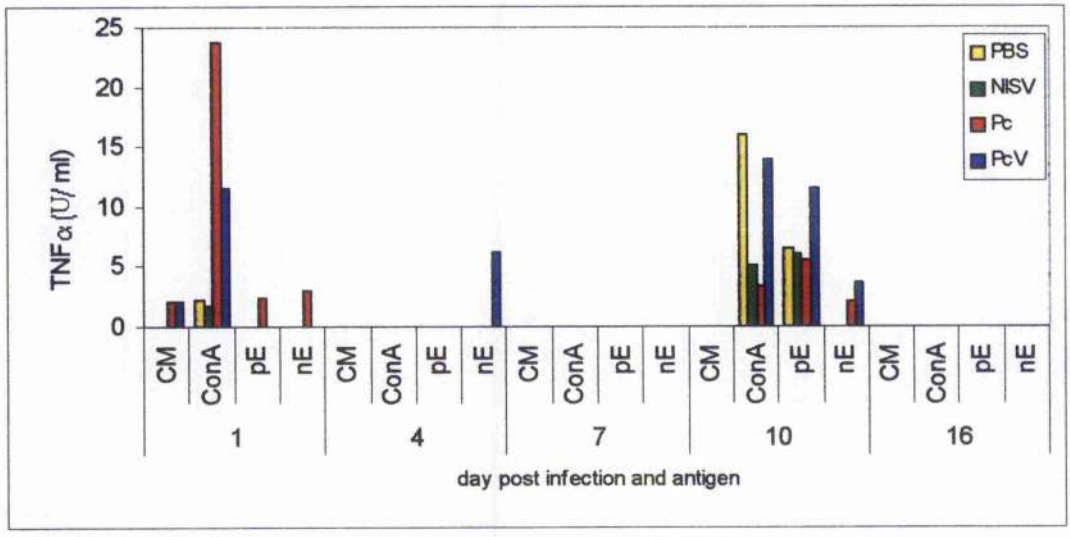


Figure 4.5: TNFα concentration in 4 hr splenic supernatants of 10-12 week old male BALB/c mice infected with *P. c* AS. Results represent pooled samples of 2 animals per group at each time point. PBS and NISV groups received 100µl s.c. of PBS and NISV respectively on days -28 and -14. Pc and PcV received a similar treatment as PBS and NIVS groups respectively but also received $1x10^5$ pRBC i.p. on day 0. Splenic cells were exposed to the following antigens: CM; complete RPMI medium, ConA; concanavalin at 10µg/ml, P; parasitized red blood cells, N; red blood cells from non-infected donor.

Discussion:

AS mentioned earlier, TNFα levels were measured following the initial promising results obtained with pre-infection treatment of *P. c.* AS-infected mice with NISV. Plasma TNFα levels were measured in three experiments. It must be emphasized that the comparison of circulating TNFα levels and indeed other cytokines (details in the subsequent chapters) between the NISV-treated and non-treated groups was done without taking standard errors into account. The reason for this is that the samples from each group were pooled to allow the measurement of 4 cytokines (TNFα, IFNγ, IL-4 and IL-10) plus measurement of circulating NO. The total amount of plasma or serum needed for each sample, therefore, is 700 µl which was not possible to obtain from a single mouse. Ideally it would be better to study samples of individual animals of each group separately, obtain their mean and standard errors and then compare them with that of the other groups. As seen from experiment S.1, no TNFα was detected early during infection (days 1-14: results not shown). To increase the sensitivity of detecting low levels of TNFα, lower concentrations of TNFα were used to obtain the standard curve. Dropping the highest TNFα concentration in the standards from 20ng/ml-2ng/ml was indeed effective in detecting some TNFα in the subsequent two experiments (S.2, G.2). In both these experiments two peaks of TNFα were seen in the Pc group while only one was seen in the PcV group with lower TNFα levels. With the three experiments taken together it can be seen that pre-infection treatment of male BALB/c mice with NISV caused suppression of circulatory TNFα levels. This finding is in agreement with the hypothesis of the NISV suppliers (Brewer and Alexander, University of Strathclyde) who suggested that NISV suppressed TNFα production from stimulated macrophages (personal communication). Early studies on TNFα have shown a positive correlation between high TNFα levels and cachexia (Beutler *et al.*, 1985) In the results presented here, however, it can be seen that although NISV suppressed plasma TNFα levels, they did not necessarily suppress malaria-associated weight loss in the treated mice (greater weight loss occurred in the Pc group in experiments S.1 and S.2, while in experiment G.1 greater weight loss in the PcV group). Weight loss was also greater in the PcV group in experiment G.2 (no plasma TNFα levels are available for this experiment).

The discrepancy in the clinical outcome between the Pc and PcV groups was also seen in the percent parasitaemia and health status score (higher percent parasitaemia and lower score were seen in the Pc group in experiments S.1 and S.2, while in experiments G.1 and G.2, higher parasitaemia and lower score were seen in the PcV group).

The explanation for these results could be several fold. First, it could be that NISV cause a non-dose-dependent TNFα suppression leading therefore to a variable degree of TNFα suppression and consequently a variable clinical outcome. As mentioned before, TNFα has a dual protective/pathological effects in malaria depending on its levels (Curfs *et al.,* 1993). It therefore becomes possible to think that if NISV suppress TNFα production by a certain amount, the mice would lose less weight (Beutler *et al.*, 1985, Pekala *et al.,* 1983, Torti *et al.*, 1985) while if too much TNFα was suppressed then the mice would succumb to the disease. In experiments S.1 and S.2, TNFα was measured as ng/ml while in G.1, it was measured as U/ml. This makes it difficult to compare the levels of TNFα in the two experiments and the amount of suppression induced by the NISV. One would, however, expect the mice with very little TNFα to die without exhibiting much of the clinical features which are thought to be mainly due to high levels of TNFα (Beutler *et al.*, 1995). As mentioned in the previous chapter, four animals were found dead following administration of the second dose of the NISV without any clinical signs of injury and without any signs of illness. Their death could not be attributed to any cause. If NISV do cause an uncontrollable suppression of TNFα, then their death could be attributed to the NISV causing suppression of TNFα to the extent that mice could not survive and also did not show any signs of illness.

Another explanation could be that although TNFα was suppressed, TNFβ which shares many of TNFα properties and its receptors (Hohman *et al.*, 1990), could have been unaffected by the NISV treatment and so produced the pathological effects. Indeed lymphotoxin has been shown to be induced by disrupted *P. falciparum*-infected red cells by mononuclear cells *in vitro* (Ferrante *et al.*, 1990). Furthermore, TNFα is known to exist in a membrane bound form (Decker *et al.*, 1987, Kriegler *et al.*, 1988) and although the circulatory levels were suppressed, it could be that the

membrane-bound form was not altered and so pathological effects of TNFα were produced by this membrane-bound form.

A third explanation is that the difference seen between the two groups with regards to the clinical features was not genuine but merely reflected biological variation among the infected animals. Indeed when the experiments were taken together, no difference was seen in their weights, parasitaemia and mortality (Chapter 3). This would, therefore, mean that TNFα suppression did not produce a significant impact on the clinical outcome of the disease in these mice. It would, however, be interesting to test the NISV effects on conditions in which suppression of TNFα by means of anti-TNFα monoclonal antibodies or soluble TNFR have resulted in improved clinical outcome (Grau *et al.*, 1989, Garcia *et al.*, 1995).

TNFα was also measured in whole blood samples. The results showed no significant difference between the two groups except on days 12 and 14 when TNFα was detected in one group only. Since this experiment was conducted once only and since TNFα measurements in whole blood have never been reported before, it becomes difficult to interpret these results and further work would be required. It has been reported, however, that damaged red cell caused the release of TNFα from human peripheral blood mononuclear cells. The TNFα-inducing activity of damaged malaria-infected erythrocytes was found to be 200 times higher than naïve erythrocytes. (Bate *et al.*, 1994). The TNFα detected in lysed whole blood here could, therefore, be explained by the findings of Bate and colleagues.

The ex-vivo splenic TNFα also showed suppression in TNFα in the PcV group on day 1 post-infection. TNFα was, however, detected only in experiment G.1 while no TNFα was detected in the splenocyte supernatants of experiment G.2 which makes it difficult to interpret the results of experiment G.1. The early production of TNFα seen in both groups could be due to IFNγ activation of macrophages since early IL-12 has been reported to induce IFNγ production from NK cells (Handa *et al* 1983, Trinchieri *et al.*, 1995).

In view of the above results and the results presented in the previous chapter, it can be concluded that pre-infection treatment of *P. c.* AS-infected male BALB/c mice with NISV causes apparent plasma TNFα suppression but that this suppression does not correlate with clinical outcome of the animals. Further work followed to elucidate whether this apparent suppression was genuine or a mere reflection of chance (details are in the subsequent chapters). Also, had adequate amount of samples been available to allow measurement of standard errors, the question of whether NISV suppressed TNFα levels would have been answered better.

Chapter 5

Effects of pre-infection administration of NISV on the IFNγ levels in male BALB/c mice infected with *P. chabaudi chabaudi* AS

Introduction

In the previous chapter, it was seen that pretreatment of *P. c.* AS-infected male BALB/c mice was associated with a suppression of circulating plasma TNFα. IFNγ is known to activate macrophages to produce TNFα (Grau *et al.*, 1989). In order to determine the mechanisms of NISV-induced suppression of TNFα, circulating IFNγ levels were also measured, results of which are presented here.

Interferon gamma (IFNγ) :

IFNγ, a 34kDa homodimer glycoprotein (Greenlund *et al.*, 1992) was discovered by two independent groups and so initially was given two names reflecting its biological activities namely a) type II interferon and b) macrophage activating factor (MAF) (reviewed by Billiau 1996). IFNγ was first identified as an antiviral agent in the supernatants of leukocytes cultures stimulated by the mitogen phytohaemagglutinin (Wheelock, 1965). Subsequently it was realized that antiviral activity was exhibited by what are now known as IFNα, IFNβ and IFNω while IFNγ had other distinct properties. IFNγ was found to have a 10-100 fold lower specific antiviral activity compared with IFNα or IFNβ but a 100-10,000 fold higher immunomodulating activity (Pace *et al.*, 1985).

Both humans and mice contain only a single IFNγ gene that is located on chromosomes 12 and 10 respectively (Naylor *et al.*, 1983, Naylor *et al.*, 1984). The size, amino acid sequence and glycosylation of IFNγ, are well conserved among various animal species (reviewed by Billiau 1996). IFNγ is made up of two elongated 17 kDa subunits that are held together in an antiparallel configuration with the amino terminus of each chain being juxtaposed to the carboxy terminus of the opposing chain. This structure allows IFNγ to bind a pair of identical receptor peptides (Walter *et al.*, 1995).

The main source of IFNγ is activated Th1 $CD4^+$ T cells (Mosmann and Coffman, 1989), with those cells expressing CD30 membrane antigen as the principal subset producing IFNγ (Alzona *et al.*, 1994). This, therefore, means that any factor that regulates the polarization of $CD4^+$ cells into Th1 and Th2 cells affects the production of IFNγ. IL-12, for instance favours the development of naive $CD4^+$ T cells into Th1 subset while IL-4 inhibits this process (Seder *et al.*, 1993). IFNγ production from $CD4^+$ T cells is induced by stimuli such as specific antigen in the context of MHC molecules, by direct stimulation of the TCR with anti-CD3 antibodies or with mitogens such as phytohaemagglutinin or concanavalin A (Gajewski *et al.*, 1989). IFNγ production by Th1 cells requires the cooperation of accessory cells, which are mostly mononuclear phagocytes. These cells are needed both to secrete regulatory cytokines like TNFα and IL-12 (Rothe *et al.*, 1993, Chan *et al.*, 1992) and also to make contact with lymphocytes through intercellular adhesion molecules (Wingren *et al.*, 1993). IL-10 inhibits IFNγ production by Th1 cells (Fiorentino *et al.*, 1989, Moore *et al.*, 1993) not through direct action on the Th1 cells (Fiorentino *et al.*, 1991) but by inhibiting the expression of these accessory molecules (reviewed by Farrar and Schreiber 1993).

Following stimulation of Th1 cells, IFNγ mRNA is first detectable at 6-8 hr, peaks by 12-24 hr and declines slowly thereafter. IFNγ itself is first detected in the extracellular environment 8-12 hr post-stimulation and peaks at 18-24hr (reviewed by Farrar and Schreiber, 1993). In *in vitro* systems, it is possible to measure the released amount of IFNγ long (up to 5 days in my experience) after activation of T cells (Langer and Pestka, 1988) but in *in vivo* systems, IFNγ can be detected in the human or murine circulation transiently due to its rapid removal from the plasma because of the abundance of IFNγ receptors (reviewed by Farrar and Schreiber, 1993).

Other sources of IFNγ include activated NK cells (Handa *et al.*, 1983, Perussia, 1991). Mitogens and microbial products have been shown to induce IFNγ production from naïve NK cells *in vitro* and *in vivo* (Bancroft *et al.*, 1987, Bancroft *et al.*, 1989) with the help of TNFα, IL–12 and macrophages (Bancroft *et al.*, 1989, Bancroft *et al.*, 1992, Trinchieri, 1995). IL-1β was shown to be needed for IL-12 to stimulate IFNγ

production by NK cells (Hunter *et al.*, 1995). Also, Gram-positive bacterial preparations such as *Staphylococcus aureus* components have been shown to stimulate IFNγ production from NK cells *in vitro* (Yoshihara *et al.*, 1993). $CD8^+$ cytotoxic T cells of the type 1 cytokine phenotype are another source of IFNγ. Mouse splenic $CD8^+$ T cells in the presence of IFNγ or IL-12 plus anti-IL-4 antibodies, differentiate into $CD8^+$ T cells producing Th1 cytokines (IL-2 and IFNγ) (Sad, Macrotte and Mosmann, 1995). IFNγ was also shown to be constitutively expressed by resting mouse peritoneal macrophages and to upregulate its gene expression in them (Di Marzio *et al.*, 1994).

IFNγ interacts with a specific cell surface receptor of high affinity (Finbloom *et al.*, 1985), which is ubiquitously expressed on all nucleated cells (Langer and Pestka., 1988, Valente *et al.*, 1992). The receptor consists of a 90kD α chain which is the ligand-binding unit (Finbloom *et al.*, 1985, Aguet *et al.*, 1988) and a 314 amino acid β chain which acts as an accessory chain required for signal transduction (Hemmi *et al.*, 1994, Masters *et al.*, 1995). The α chain is encoded by chromosomes 6 and 10 in humans and mouse respectively (Pfizenmatier *et al.*, 1988, Mariano *et al.*, 1987) and the β subunit is encoded by chromosomes 21 and 16 in human and mouse respectively (Soh *et al.*, 1994, Hemmi *et al.*, 1994).

The signal transduction pathway starts with IFNγ interaction with two α chains inducing α chain dimerization. The two β chains then associate with the IFNγ–α chain complex leading to phosphorylation and activation of JAKs. Further steps of phosphorylation and activation occur leading finally to the activation of STAT1α that translocates into the nucleus and binds to IFNγ activation region on the DNA and initiates transcription. Once signalling has occurred, the IFNγ–α chain complex is internalized and dissociates with the free IFNγ eventually being degraded in the lysosome and the receptor recycling back to the surface of the cells (Anderson *et al.*, 1983).

IFNγ is a pleotropic cytokine with many functions. Its main cellular target is the monocyte/macrophage cell. IFNγ affects the differentiation of immature myeloid cells

into mature monocytes and activates macrophages by both upregulating the expression of MHC II molecules (Basham and Merigan, 1983, Cao *et al.*, 1989) and increasing the levels of several enzymes needed for antigen processing (Allen and Seed, 1989). IFNγ induces protein 10, which is a chemo-attractant for human monocytes and T cells and promotes T cell adhesion to endothelial cells (Taub *et al.*, 1993)

IFNγ increases intracellular killing of parasites (Nathan *et al.*, 1983), enhances phagocytosis (Ockenhouse *et al.*, 1984, Shear *et al.*, 1989), enhances tumor killing (Weinberg and Larrick, 1987) and induces production of TNFα (Grau *et al.*, 1989, Murray *et al.*, 1985) and that of reactive oxygen intermediates (Nathan *et al.*, 1983, Kamijo *et al.*, 1993a). Moreover, it enhances the cytolytic activity of NK cells (Gidlund *et al.*, 1978). IFNγ was also reported to regulate the differentiation of mouse splenic $CD8^+$ T cells (Sad, Marcotte and Mosmann, 1995).

Several studies have reported a role for IFNγ in protection against malaria. IFNγ-treated monocytes and monocyte-derived macrophages killed *P.falciparum in vitro* and resulted in the appearance of crisis forms (Ockenhouse *et al.*, 1984, Gyan *et al.*, 1994). This lethal action was inhibited by addition of anti-IFNγ antibodies to the culture (Ockenhouse *et al.*, 1984). It was also shown that the parasite growth inhibition was due to production of NO since addition of the NO synthase inhibitor, LNMMA to the macrophages in culture reduced parasite growth inhibition (Gyan *et al.*, 1994). Daily treatment of *P chabaudi adami* infected CBA/CaH mice with murine recombinant IFNγ (rIFNγ) starting from day –1 produced a dose-dependent delay in the onset of a patent parasitaemia, and an earlier appearance of crisis forms (Clark *et al.*, 1987). In mice infected with the lethal strain of *P. yoelii* (17X), treatment with rIFNγ resulted in a significantly lower peak parasitaemia and increased survival (Shear *et al.*, 1989). IFNγ was seen to increase transiently in the serum of mice infected with *P. c.* AS about 3-4 days prior to primary peak parasitaemia (Slade and Langhorne, 1989). Moreover, IFNγ production *in vitro* from splenocytes of the resistant C57Bl/6 mice infected with *P. c.* AS was seen to peak 2-3 days prior to primary peak parasitaemia (Langhorne *et al.*, 1989, Stevenson *et al.*, 1990). Treatment of *P. c.* AS-infected resistant C57Bl/10ScN mice with anti-IFNγ antibodies resulted in

a greater parasitaemia 2 days before and at peak parasitaemia (Stevenson *et al.*, 1990). Furthermore, treatment of *P. berghei*-infected C57Bl/6 mice with rIFNγ prevented the development of experimental cerebral malaria (Curfs *et al.*, 1993). Treatment of CBA/JNCrj mice infected with the attenuated *P. berghei* (PbXAT) strain with anti-IFNγ antibodies led to failure of resolution of the infection associated with suppression in malaria specific IgG2a levels (Waki *et al.*, 1995). The above studies, therefore, indicate a protective role for IFNγ in several host-parasite combinations. Subsequent studies have also confirmed this protective role but in addition showed that despite its protective role, IFNγ was not essential for clearance of the infection. *P. c.* AS-infected IFNγR-KO mice have a similar level of primary parasitaemia but fail to reduce it to subpatent levels (Balmer *et al.*, 2000), have a greater mortality and a higher second peak of parasitaemia that their wild type counterparts (Balmer *et al.*, 2000, Favre *et al.*, 1997). Both wild type and IFNγR deficient mice, however, were equally protected upon re-infection (Favre *et al.*, 1997). The above studies clearly indicate that IFNγ deficiency impairs immunity against primary parasitaemia but does not abrogate it. In addition, transient increase in serum IFNγ levels were detected in both *P. c.* AS-infected resistant BALB/c (H-2d) and susceptible DBA/2 (H-2d) mice (Slade and Langhorne, 1989) indicating that IFNγ is not the determining factor for host susceptibility or resistance to malaria infection. Other studies, however, have shown that early splenic IFNγ production (24hr post-infection) was the difference between two lethal and two non-lethal malaria infections (de Souza *et al.*, 1997).

It must be emphasized, however, that IFNγ was also shown to mediate harmful effects. Treatment of *P. berghei*-infected CBA/Ca mice with anti-IFNγ monoclonal antibodies no later than day 4 post-infection markedly reduced the incidence of cerebral malaria and the associated cerebral lesions of capillary plugging by monocytes, lymphocytes and parasitized red blood cells (Grau *et al.*, 1989).

Apart from its role in blood stage infection, IFNγ was also shown to play a role in immunity against exoerythrocytic stages. IFNγ has been shown to inhibit *P. falciparum* sporozoite development in hepatocytes (Mellouk *et al.*, 1987) and the exoerythrocytic forms of *P. berghei in vitro* (Ferreira *et al.*, 1986). Systemic administration of IFNγ protects rhesus monkeys against a *P. cynomolgi* B sporozoite

challenge (Maheshwari *et al.*, 1986). Furthermore, the immunity observed in mice immunized with irradiated sporozoites was abrogated upon treatment of the mice with neutralizing anti-IFNγ antibodies (Schofield *et al.*, 1987c)

In the studies presented in this thesis, male BALB/c were infected with *P. c.* AS and a group was pretreated with NISV. The animals were followed up clinically (see Chapter3), and in terms of their cytokine production, TNFα (Chapter 4), IL-4 (Chapter 6) and IFNγ, that is presented here. The aim was to investigate whether NISV-induced suppression of TNFα (details in Chapter 3) occurred through mechanisms involving IFNγ.

Results

The work described here represents the results of 4 experiments in which male BALB/c mice were infected with *P. c.* AS and treated with NISV pre-infection (days -28 and -14). IFNγ levels were measured in splenic supernatants and plasma samples. Also a new method of measuring IFNγ in lysed whole blood was tried and is described below.

5.1. Effects of NISV on the plasma levels of IFNγ

Inbred male BALB/c mice (10, 20 and 20 per group in experiments S.1, S.2, and G.1 respectively) were infected i.v. with $1x10^5$ pRBCs of *P. chabaudi* AS on day 0. Two groups had received 100µl of PBS or NISV on days -28 and -14 subcutaneously and were referred to as Pc and PcV respectively. Two control groups were injected with 100µl of PBS or NISV and referred to as PBS and NISV respectively. Equal volumes of blood were collected from the tail veins of 5 animals (experiments S.1, S.2) or through cardiac puncture of 2 animals (experiment G.1) per group at each time point indicated into heparinized capillary tubes. Blood samples were centrifuged at 13000 rpm (4000g) for 5 minutes and plasma samples of each group were pooled and stored at –20°C until further use.

In experiment S.1 significantly higher amounts of IFNγ was found in the plasma of the NISV-treated group (PcV) on days 13, 15, 17, 25, and 40 post-infection, while higher levels were found in the untreated group (Pc) on days 19, 21, 23, 29, 34 and 42 (Figure 5.1). Peak parasitaemia in this experiment was on day 9 and the trough of weight loss on days 11 and 10 in the Pc and PcV groups respectively (see Appendix 2). In experiment S.2, significantly higher IFNγ levels were found in the plasma of the PcV group on days 15, 16, 19 and 21 post-infection, while higher levels were seen in the Pc group on days 9 and 14 post-infection (Figure 5.2). Peak parasitaemia was on day 8 and trough of weight loss was on day post-infection (see Appendix 2). As mentioned previously, the PcV group had a less severe disease than the Pc group in these two particular experiments with regards to level of parasitaemia, degree of weight loss and health status score (see Appendix 2 for results of individual experiments). In experiment G.1, the animals were followed up for 16 days only when they had recovered clinically from the primary parasitaemia. No IFNγ was detected from day 1-7 except in the PBS group on day 1. IFNγ peaked on day 9 post infection in both Pc and PcV groups (Figure 5.3), which coincided with day of primary peak parasitaemia. No significant difference was seen between groups Pc and PcV at any time points. In this particular experiment, the Pc group exhibited a less severe disease than the PcV in terms of parasitaemia levels, and degree of weight loss (see Appendix 2).

With the three experiments taken together, it can be seen that IFNγ was never found in the plasma of the infected animals early in the infection and prior to the primary peak of parasitaemia. It can also be seen that there was no consistent difference in the plasma IFNγ levels between the NISV-treated and untreated groups. Plasma IFNγ levels in the PBS and NISV groups were measured in one experiment only (G.1) and there were found to be very low (100 U/ml) and there was no difference between the two groups.

5.2. Effects of NISV on the IFNγ levels measured in lysed whole blood

Twenty five inbred male BALB/c mice per group were infected as described above. Blood samples were collected from either five animals from tail vein (days 3, 4, 5, 7, 12, 15) or two animals by cardiac puncture per group (days 6, 8, 10, 11, 14, 17, 21) at each time point. Samples from each group were pooled together and stored at -20°C until further use. ELISAs were done according to the standard method described in material and methods. It was noted, however, that by leaving the plate with blood samples at 4°C over night and completing the rest of the steps of ELISA on the following day, IFNγ (and other cytokines) were detected. Without this modification in the ELISA steps, no cytokines were detected in the whole blood samples.

Two important observations were made in IFNγ levels in whole blood. First, it was noted that IFNγ was seen early during infection (from day 3-day 8) while during peak parasitaemia it was undetectable but it reappeared afterwards, albeit at much lower levels. The second point is that IFNγ was slightly higher in the PcV group on days 5 and 7 post-infection (Figure 5.4). The NISV-treated group exhibited a more severe disease in this experiment, with regards to level of parasitaemia, degree of weight loss and health status score (see Appendix 2). IFNγ measurements in the lysed whole blood of the PBS and NISV groups were done at one time point only (day 21 post-infection). Low levels of IFNγ (approximately 48 U/ml) were detected in both groups. Whole blood samples were used in one experiment only (G.2) and so no comparison can be made with other results.

5.3. Effects of NISV on IFNγ levels in splenocyte supernatants

IFNγ in splenocyte supernatants of *P. c* AS-infected mice was measured in two experiments, both of which were conducted at Glasgow University. Spleens of 2-3 animals per group were removed aseptically at each time point (days 1, 4, 7 and 10 and days 6, 8, 10, 11, 14, 17 and 21 post-infection in experiments G.1 and G.2 respectively). Splenocytes of each group were pooled together, exposed to various stimulating or control agents including normal medium, ConA, non-infected mouse

red blood cells (nE) and parasitized mouse red blood cells (pE), and incubated for 72 hr at 37°C in 5% CO_2 in air. Supernatants were collected and stored at -20°C until further analysis.

IFNγ peaked on day 4 post-infection in experiment G.1 to all stimulants used. Low IFNγ levels were also detected in response to ConA on day 6. IFNγ levels were slightly higher in the NISV-treated group on day 4 while on day 6 the PcV group had a slightly lower levels than the Pc group. Low levels of IFNγ were detected in the splenocyte supernatants of PBS and NISV at all time points. In the second experiment (G.2), high IFNγ levels were seen on days 6, 17 and 21 post-infection (Figure 5.6.). In experiment G.2, IFNγ measurements in the splenocyte supernatants of the PBS and NISV groups were done at one time point only (day 21 post-infection). IFNγ in the NISV group was approximately half of the levels in the PBS splenocyte supernatants.

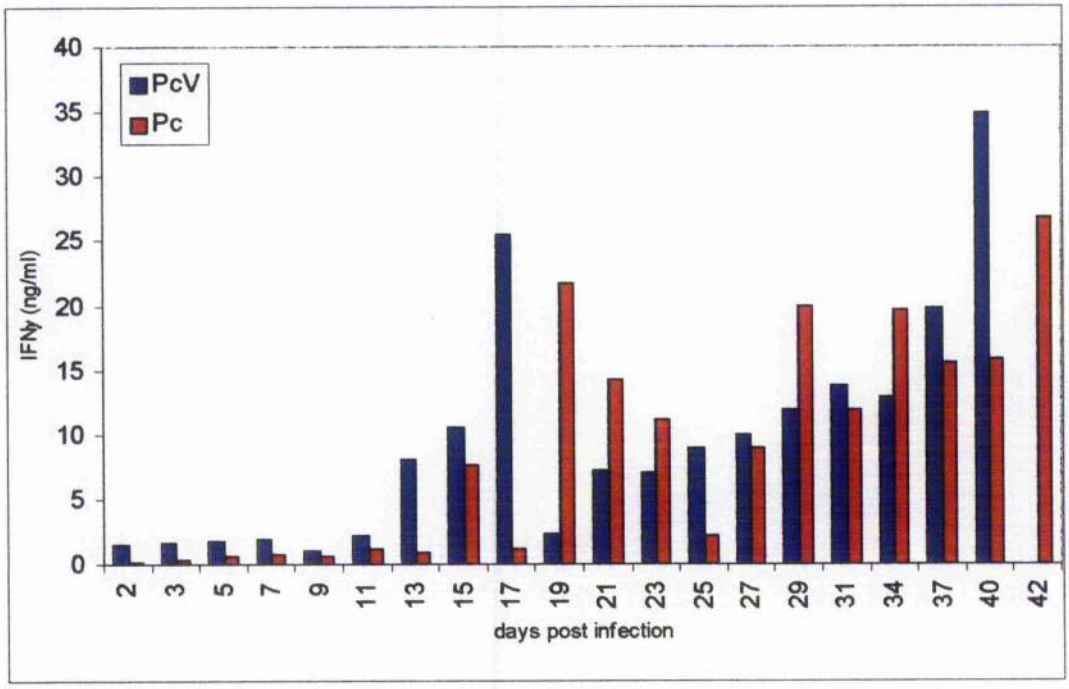


Figure 5.1. (experiment S.1): Plasma IFNγ levels of 10-12 week old male BALB/c mice infected with *P chabaudi* AS. Both groups (Pc and PcV) were injected i.v. with $1x10^5$ pRBC on day 0 and received 100µl s.c. of PBS and NISV respectively on days -28 and -14. Results represent pooled samples of 5 animals per group at each time point.

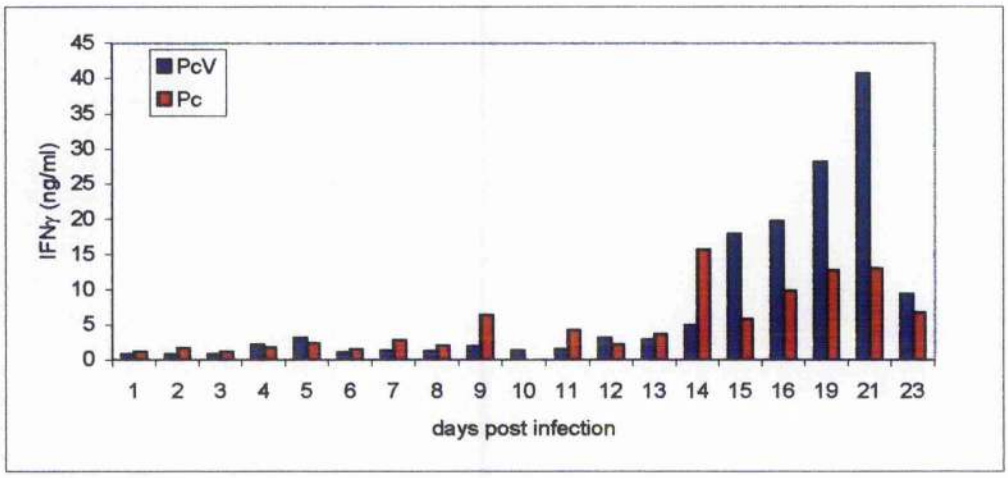


Figure 5.2. (experiment S.2): Plasma IFNγ level 10-12 week old male BALB/c mice infected with *P chabaudi* AS. Both groups (Pc and PcV) were infected i.p. with $1x10^5$ pRBC on day 0 and received a s.c. inoculation of 100µl of BPS or NISV respectively on days -28 and -14. Results represent pooled samples of 5 animals per group at each time point.

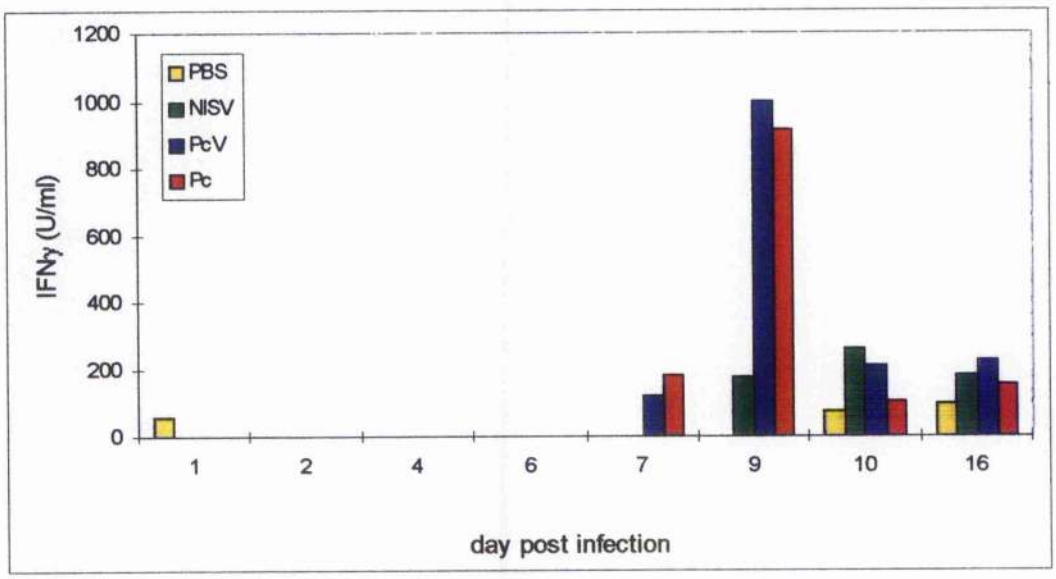


Figure 5.3. (experiment G.1): Plasma IFNγ levels of 10-12 week old male BALB/c mice infected with *P. chabaudi* AS. Groups Pc and PcV were injected i.v. with $1x10^5$ pRBC on day 0 and received s.c. inoculation of 100µl of PBS or NISV respectively on days -28 and -14. Groups PBS and NISV were only injected s.c. with 100µl of PBS or NISV respectively on days -28 and -14.

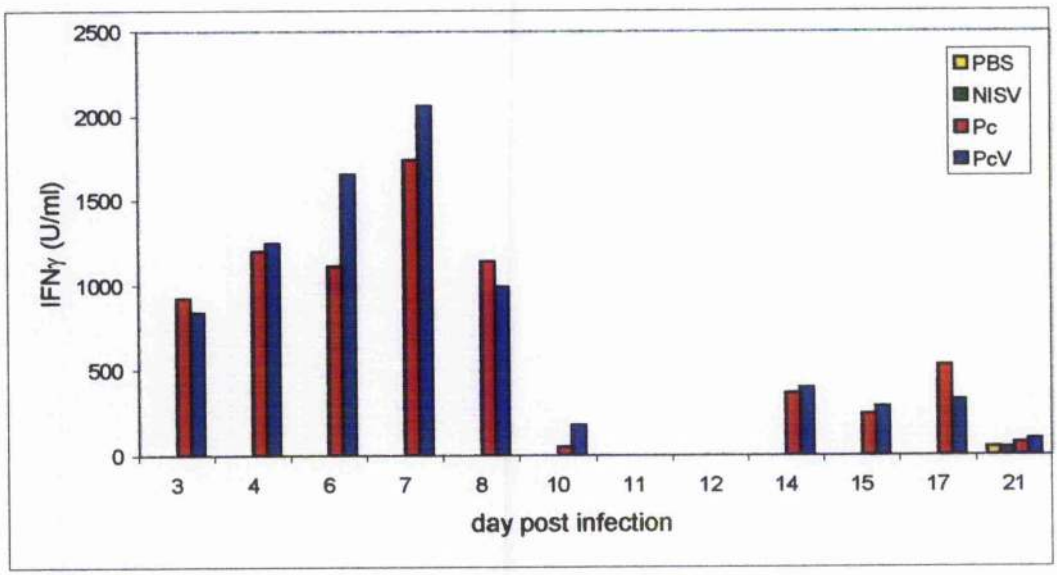


Figure 5.4. (experiment G.2): IFNγ levels in whole blood of 10-12 week old male BALB/c mice infected i.p. with *P chabaudi* AS. Groups Pc and PcV were infected with $1x10^5$ pRBC on day 0 and received 100µl s.c. of PBS and NISV respectively on days -28 and -14. PBS and VISV groups wre injected with 100µl of PBS or NISV respectively on days –28 and –14.

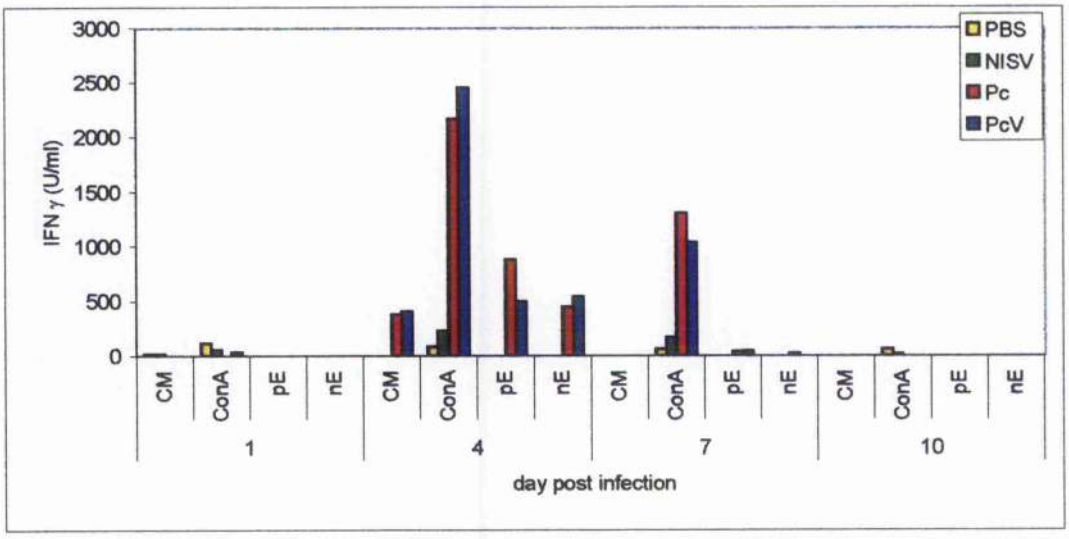


Figure 5.5. (experiment G.1): 72hr IFNγ levels in splenocyte supernatants of male BALB/c mice infected with $1x10^5$ pRBC. Pc, PcV groups were infected with *P. chabaudi* AS i.p. on day 0 and had received 100µl of PBS and NISV respectively s.c. on days -28 and -14. PBS and NISV are 2 control groups, received 100µl PBS or NISV respectively on days -28 and -14. CM (complete RPMI medium), ConA (concanavalin at 10µg/ml), nE (non-infected mouse red blood cells), pE (parasitized mouse red blood cells). Results represent spleens of 2 animals per group at each time point.

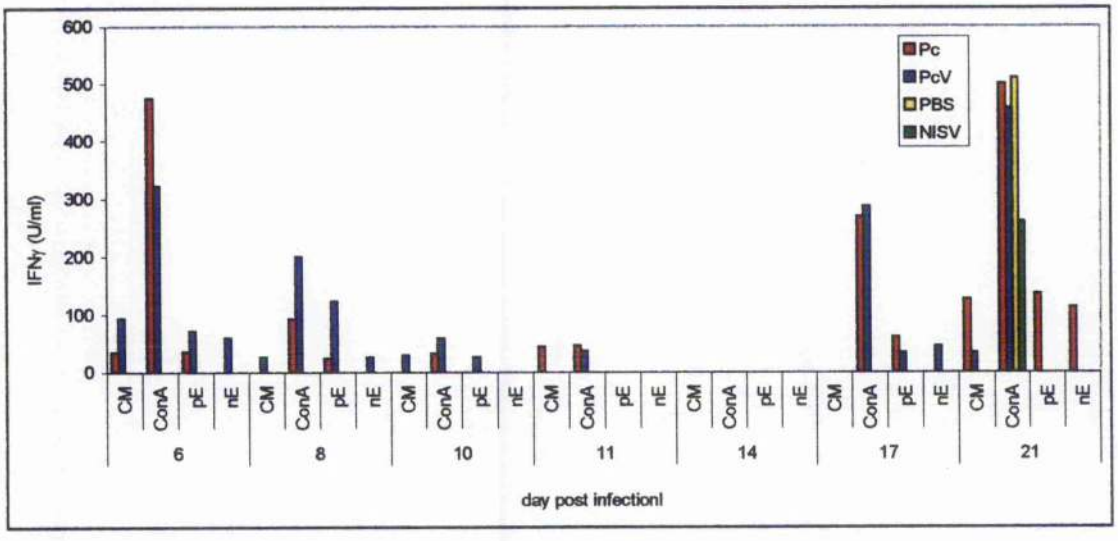


Figure 5.6. (experiment G.2): IFNγ levels in 72hr splenic supernatants of *P. chabaudi* AS-infected male BALB/c mice. Pc and PcV groups were infected i.p. with $1x10^5$ pRBC on day 0. Pc and PBS received 100µl of PBS on days –28 and –14. Groups PcV and NISV received 100µl of NISV on days –28 and –14. CM; complete RPMI medium, ConA; 10µg/ml concanavalin, pE; parasitised red blood cells and nE; non-infected red blood cells.

Discussion:

The work done in the previous chapter showed that pretreatment of *P. c* AS-infected BALB/c mice with NISV led to suppression of the circulating TNFα levels. Since IFNγ has been reported to stimulate TNFα production by macrophages (Murray *et al.*, 1985, Grau *et al.*, 1989), IFNγ was also measured in these mice to see if NISV-induced suppression of TNFα occurred through mechanisms involving IFNγ. The results presented here are discussed in the context of the effects of NISV on IFNγ levels and the relation between IFNγ levels and disease severity.

Plasma IFNγ levels in the three experiments (S.1, S.2 and G.1) were not elevated early during infection. This is in contrast to results reported by other groups which showed a transient increase in IFNγ 2-3 days prior to primary peak parasitaemia which correlated with protection in *P. c.* AS infections in female C57Bl/6, BALB/c ($H\text{-}2_d$) and DBA ($H\text{-}2_d$) mice (Slade and Langhorne, 1989, Langhorne *et al.*, 1989). It is possible, however, that IFNγ was not detected because blood was collected from the animals after IFNγ had disappeared from the circulation by binding to its widely distributed receptors (reviewed by Farrar and Schreiber, 1993). Also in experiments S.1, S.2 and G.1 plasma IFNγ levels were found to increase in both Pc and PcV groups later during infection following the primary peak parasitaemia. In all these experiments no consistent difference was seen between the two groups in their IFNγ levels in relation to the use of NISV or in relation to disease severity (in all experiments, the PcV group exhibited a less severe disease in terms of parasitaemia levels, degree of weight loss and health status score). In experiment G.1, however, IFNγ was found to peak on day of peak parasitaemia in both groups without any significant difference in the two groups. As mentioned earlier, in this particular experiment, the Pc group exhibited a less severe disease in terms of weight loss, percent parasitaemia and health status score. With the three experiments taken together, it can be said that the plasma IFNγ levels did not reflect disease severity nor were they altered by the use of NISV.

High levels of splenic IFNγ were seen on days 4 and 7 in experiment G.1 (peak parasitaemia occurred on days 8 and 9 in the PcV and Pc groups respectively) and days 6 and 8 in experiment G.2 (peak parasitaemia was on day 9 and 10 in the Pc and PcV groups respectively). It must be noted, however, that the amount detected on day 4 in experiment G.1 (Figure 5.5) is much higher than that detected on day 7 of experiment G.2 (Figure 5.6). Since a lower level was also detected on day 6 in experiment G.1, this could well mean that a much higher level could have been missed on day 4 in experiment G.2. The high levels of IFNγ seen prior to peak parasitaemia agree with the previous findings of splenocytes producing IFNγ 2-3 days prior to peak parasitaemia (Langhorne *et al.*, 1989, Stevenson *et al.*, 1990). No correlation, however, was seen between the use of NISV and the *ex-vivo* splenic levels of IFNγ.

An important observation made during this work on measurement of cytokines by ELISAs is that IFNγ could also be measured in lysed whole blood samples. This, to date, has not been reported and was thought of in an attempt to save time. Again no significant difference was seen between the NISV-treated and untreated groups. Moreover, no significant difference was seen between the two control groups PBS and NISV. Any differences seen in the IFNγ levels between the two groups (Pc and PcV) in plasma, blood or splenocyte supernatants could simply reflect normal variable activity between different cells at any single time point.

In view of the above results, it is clear that pre-infection treatment with NISV does not affect IFNγ levels in the treated mice. This, therefore means that the NISV-induced suppression of TNFα (reported in the previous chapter) does not occur through IFNγ. This is in agreement with the hypothesis suggested by the investigators of the NISV (Dr. Brewer, Division of Immunology, Infection and Inflammation, Western Infirmary, Glasgow and Alexander, Strathclyde University) who suggested that NISV acted on the macrophages directly to suppress TNFα production (personal communication).

An interesting observation made in the IFNγ levels in whole blood samples was the fact that high levels were seen early in the infection and prior to peak parasitaemia.

This is in contrast to the plasma levels, which did not increase until after peak parasitaemia. It is difficult to comment on these results since no previous studies reported using whole lysed blood for measurements of cytokines and since this work was done only once. It would useful to repeat it again before drawing any conclusions.

The conclusion arrived at from work done in this chapter is that since it was seen from the last chapter that pre-infection treatment of animals with NISV led to suppression of the circulating TNFα levels regardless of the clinical outcome and since it is reported that IFNγ stimulates the production of TNFα from macrophages, it seems that the NISV do not suppress TNFα levels by mechanisms related to the IFNγ levels. It seems that the NISV act on the macrophages and are probably engulfed by them and suppress their ability to secrete TNFα in response to stimuli such as malaria antigens or IFNγ.

Chapter six

Effects of pre-infection treatment with NISV on the levels of IL-4 in male BALB/c mice infected with *Plasmodium chabaudi chabaudi* AS.

Introduction:

Following my work on the effects of the administration of NISV pre-infection on the subsequent course of *Plasmodium chabaudi* AS infection in male BALB/c mice, it was considered important to measure not only the Th1 response but also the Th2 response of the $CD4^+$ T cells in these mice. The importance of this was two fold, first to see if NISV did have any immunoregulatory effects on the Th1/Th2 polarization and secondly to find the mechanism of TNFα suppression. The Th2 cytokines chosen for measurement were IL-4 and IL-10. In the event the standards in the IL-10 ELISA kit were found to be unreliable by others and myself in the department and therefore only IL-4 results are included in this chapter.

IL-4:

IL-4 is a 20kDa glycoprotein, initially identified as a B cell growth factor found in the supernatants of mouse thymoma EL4 cells (Howard *et al.*, 1982) and so was named B cell stimulatory factor-1(BSF-1). Human and mouse IL-4 are 153 and 140 amino acid polypeptides respectively and are encoded by a gene on chromosome 5 in humans and 11 in mouse. The gene is part of a cytokine complex including genes for IL-3, IL-5 and GM-CSF (Paul, 1991).

IL-4 is produced mainly by $CD4^+$ Th2 cells. Mouse Th2 cells are a subset of T helper cells characterized upon activation by production of IL-4, IL-3 and mast cell growth factor (Mosmann *et al.*, 1986). Other cellular sources of IL-4 include mast cells (Brown *et al.*, 1987), γδ T cells (Ferrick *et al.*, 1995), CTL cells (Seder *et al.*, 1992), a population of $CD4^+$ $NK1.1^+$ NKT cells (Yoshimoto *et al.*, 1994) and non-B non-T cells (NBNT) (Helmby *et al.*, 1998).

IL-4 exerts its effects by binding to complementary receptors on cell surfaces. IL-4 has two different types of receptors, classical and alternative. The classical one is expressed predominantly on haematopoetic cells and consists of a 140 kDa α chain and a 64 kDa γ chain, which is shared by the IL-2 receptor (Russell *et al.*, 1993, Kondo *et al.*, 1993). Stimulation of this receptor leads to the phosphorylation of JAK3

which in turn stimulates the transcription factor STAT6 (Murata *et al.*, 1998, Murata *et al.*, 1999). The alternative receptor, on the other hand, is predominantly expressed on non-haematopoetic cells and consists of the IL-4Rα chain and IL-13R α chain (Matthews *et al.*, 1995, Obiri *et al.*, 1995). Stimulation of this receptor leads to the activation of JAK2 which also leads to the activation of STAT6. IL-4 receptors are widely spread, being expressed on B and T cells, mast cells, macrophages, cells of mycloid and erythroid lineage and a variety of non-haematopoetic cells such as stromal cell lines from bone marrow, spleen thymus and brain (Ohara *et al.*, 1987, Park *et al.*, 1987).

IL-4 has multiple functions in different arms of the immune system. As mentioned previously it is involved in the growth and differentiation of B cells. IL-4 stimulation of B cells leads to upregulation of MHC class II molecules (Noelle *et al.*, 1984, Roehm *et al.*, 1984), IL-4 receptor and CD23 (FcεRIIb) expression ((Defrance *et al.*, 1987, Hudak *et al.*, 1987). It also leads to class switch in immunoglobulin production from IgM into the production of IgG1 (Vitetta *et al.*, 1985, Sideras *et al.*, 1985) and IgE (Coffman *et al.*, 1986).

IL-4 has also been shown to play a role in the growth of T cells and mast cells (Lee *et al.*, 1986, Mosmann *et al.*, 1986), granulocytes (Rennick *et al.*, 1986), megakaryocytes and erythrocytes (Peschell *et al.*, 1987). IL-4 has an important role in the polarization of $CD4^+$ T helper subsets. The environment of the $CD4^+$ T cells can influence their direction of development. Presence of IL-4 encourages the polarization towards Th2 cells (Essner *et al.*, 1989). IL-4 was also shown to enhance the proliferation of precursors of cytotoxic T cells and their differentiation into CTL (Widmer and Grabstein, 1987, Trenn *et al.*, 1988).

IL-4 was also reported to enhance antigen presentation by murine bone marrow-derived macrophages (Zlotnick *et al.*, 1987) by upregulating MHC class I and II molecules on them.

IL-4 has been described as anti-inflammatory because of its ability to suppress proinflammatory cytokines such as IL-1 and TNFα. Thus it was shown to suppress significantly TNFα release from peripheral blood mononuclear cells of patients with rheumatoid arthritis in response to LPS. Moreover, IL-4 suppressed the enhancing effect of IFNγ on IL-1 production from human peripheral blood mononuclear cells. The anti-inflammatory effect of IL-4, however, was not evident on all types of macrophages since those macrophages that were derived from the synovial fluids of same patients with rheumatoid arthritis, did not suppress their release of TNFα (Hart *et al.*, 1993). IL-4 was also reported to downregulate monocyte production of IL-1 and PGE2 (Hart *et al.*, 1989), IL-6 (Cheung *et al.*, 1990) and IL-8 (Standiford *et al.*, 1990). IL-4 also suppresses monocyte production of superoxide anions (Abramson and Gallin, 1990) and H_2O_2 (Lehn *et al.*, 1989). On the other hand, IL-4 increases the production of IL-1R antagonist (Vannier *et al.*, 1992), further suppressing pro-inflammatory activity. Recently IL-4 was also identified as a factor determining the type of eicosanoids produced by dendritic cells in response to growth factors such as stem cell factor and GM-CSF (Spanbroek *et al.*, 2001). Here IL-4 was reported to suppress the 5-lipooxygenase pathway, which leads to the production of potent mediators of inflammatory and allergic tissue reactions (Samuelsson *et al.*, 1987).

IL-4 has been found to play a role in several infectious diseases. In *L. major*-infected BALB/c mice, early production of IL-4 is associated with susceptibility to the infection, while resistance was noted in the IL-4 gene disrupted mice which produced a reduced Th2 response (Kopf *et al.*, 1996). IL-4 deficient mice, on the other hand, were found to be more susceptible during the initial phase of rapid proliferation of tachyzoites of *Toxoplasma gondii* than their wild-type counterparts (Roberts *et al.*, 1996). During the chronic phase of the infection, however, the IL-4 deficient mice exhibited less pathology.

In the case of the role of IL-4 in malaria, sequential appearance of Th1 and Th2 cells has been reported to play an important role in protection against *P. c.* AS-infected mice (Langhorne *et al.*, 1989) indicating a role for IL-4 late in the infection. Moreover, early production of IL-4 was found to correlate with susceptibility to *P. chabaudi* infection (Jacobs, Radzioch and Stevenson 1996). The mechanisms

responsible for the switch from Th1 to Th2, however, are not clear. Among mechanisms suggested for this switch, is the expansion of non-B non-T cells (NBNT) in the spleens and peripheral blood around the time of peak parasitaemia and the secretion of IL-4 by these cells (Helmby *et al.*, 1998). This IL-4 in turn shifts the Th0 differentiation into the Th2 direction, leading to further release of IL-4 (Essner *et al.*, 1989).

The role of IL-4 in *P. c* AS-infected mice was specifically investigated by generating IL-4 gene knockout mice. $CD4^+$ T cells obtained from these animals were found to be defective in generating Th2 cytokines *in vitro* (Kopf *et al.*, 1993). Using the above mice and comparing them to wild type mice infected with *P.c. c.* AS showed that the initial Th1 response was intact in the $IL\text{-}4^{-/-}$ mice and that Th2 activity such as generation of IgE was not impaired but rather delayed during infection (Kopf *et al.*, 1993). IL-4-/- mice were able to control and clear the primary parasitaemia but they had a higher recrudescence than their wild type counterparts. They were also found to have reduced mRNA levels for IL-5 and IL-6 but not IL-10 early during infection but later all these cytokines were expressed equally (von der Weid *et al.*, 1994). Other studies, however, have shown an effect on the primary parasitaemia in IL-4 deficient mice. *P. c.* AS-infected, IL-4 deficient BALB/c, B6x129 and 129SV mice all had a significantly higher primary peak parasitaemia than their wild type counterparts, but a similar rate of clearance of the primary patent parasitaemia (Balmer, 1997). In *P. chabaud adami* and *P. yoelii*-infected IL-4-deficient mice, on the other hand, the course of infection was not significantly different from the wild type counterparts (von der Weid *et al.*, 1994).

IL-4 on the other hand has been suggested to play an important role in the parasite evasion of the immune system. Pretreatment of human monocytes/macrophages with rIL-4 significantly suppressed their ability to phagocytose *P. falciparum* parasitized red blood cells *in vitro* (Kumaratilake and Ferrante, 1992). The authors suggested that *in vivo* the parasites could be inducing the production of IL-4 which would, therefore, reduce their killing by the monocytes.

The results presented in this chapter are IL-4 levels in plasma, lysed whole blood and splenocytes supernatant samples obtained from multiple experiments on *P. c.* AS-infected male BALB/c mice, a group of which was pretreated with NISV and the cytokine levels in both groups were compared. The same mice were followed up clinically with regards to weight loss, percent parasitaemia and health status score (see Chapter 3). TNFα and IFNγ were also measured (see Chapter 4 and 5 respectively).

Results:

6.1. IL-4 Levels in plasma samples:

Twenty inbred male 10-12 week old BALB/c mice were infected i.v. with 10^5 pRBC at Strathclyde University. Blood samples were collected by bleeding 5 mice at each time point (days 1-16 and days 19, 21, and 23 post-infection) from the tail vein into heparinised capillaries. Samples from each group were pooled together and stored at –20°C until further use. Samples were frozen and thawed about four times before IL-4 levels were measured with ELISA. This is because ELISA of other cytokines was done first (details of ELISA in Materials and Methods).

No IL-4 was detected in the plasma of either group until late in the disease (day 12), during the recovery phase of the primary infection as was evident from the health status score, percent parasitaemia and weight measurements. High levels of IL-4 were detected in the NISV-treated group (PcV) on days 12 (0.6μg/ml), 14 (0.9μg/ml) and 15 (1.5μg/ml). In the untreated group (Pc), IL-4 was detected at a single time point only (day 12) when the levels were approximately half (0.3μg/ml) of those in the NISV-treated group (Figure 6.1). In this particular experiment, the treated group (PcV) was the better one clinically in that the mice exhibited a lower parasitaemia and less weight loss than the untreated group (Pc) (see Appendix 2 for graphs of individual experiments).

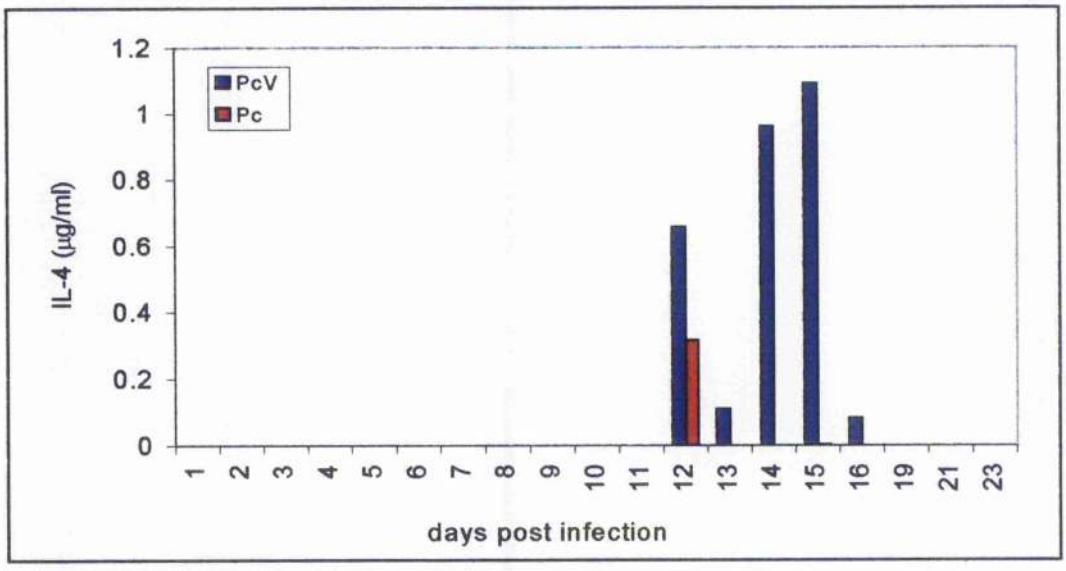


Figure 6.1. Plasma IL-4 concentration in 10-12 week old male BALB/c mice infected with *P chabaudi* AS. Pc and PcV : animals infected i.v. with 1x 10^5 pRBC on day 0 and received 100µl PBS and NISV respectively s.c. on days -28 and -14. Results represent means of pooled plasma samples of five animals run in triplicates.

6. 2. Effect of NISV on lysed whole blood levels of IL-4:

Whole blood samples were collected from 25 inbred male 10-12 week old BALB/c mice. These mice were infected with *P. c.* AS and pretreated with either NISV or PBS as before. Blood samples were obtained through two ways, either 5 mice were bled from the tail vein on days 3, 4, 5, 7, 9, 13, 15 and 19, or via cardiac puncture of 2 mice sacrificed for their spleens on days 6, 8, 10, 11, 14, 17 and 21 post-infection. The samples of each group were pooled together and stored at –20°C until further use.

IL-4 was detected at three time points in the untreated group (Pc), on days 3, 7 and 14 post-infection, with the levels being highest on day 14 (75U/ml). In the NISV-treated group (PcV), however, IL-4 was detected only once, on day 7, when it was about double the level found in the untreated group (Figure 6.2). In this particular experiment, the untreated group (Pc) was the better one clinically in terms of percentage parasitaemia and weight loss (see Appendix 2 for results of individual experiments).

6.3. Effect of NISV on the splenic levels of IL-4

The animals used here are the same as those from which lysed whole blood samples were removed i.e. 25 male BALB/c mice. Spleens of two animals per group were removed aseptically on days 6, 8, 10, 11, 14, 17 and 21 and exposed to various stimulating or control agents; concanavalinA, parasitised red blood cells (pE), non-parasitized red cells (nE) and complete RPMI medium. On day 14, however, no pE or nE were available. The splenic cells were incubated with the above stimulants for 72 hr at 37°C in 5% CO_2 in air, and their supernatants collected and stored at –20°C until analysis.

In general, higher levels of IL-4 were detected in the NISV-treated group (PcV) from day 6 to day 10 post-infection than the untreated group (Pc). The untreated group (Pc), on the other hand produced higher IL-4 levels on day 11 (around peak parasitaemia) and on day 17 post-infection.

No IL-4 was detected in the untreated group (Pc) to any stimulant on days 8, 10 and 21 post-infection (Figure 6.3). As mentioned above in this particular experiment the untreated group (Pc) exhibited a less severe disease that the NISV-treated group (PcV) in terms of percent parasitaemia and amount of weight loss (see Appendix 2 for results of individual experiments).

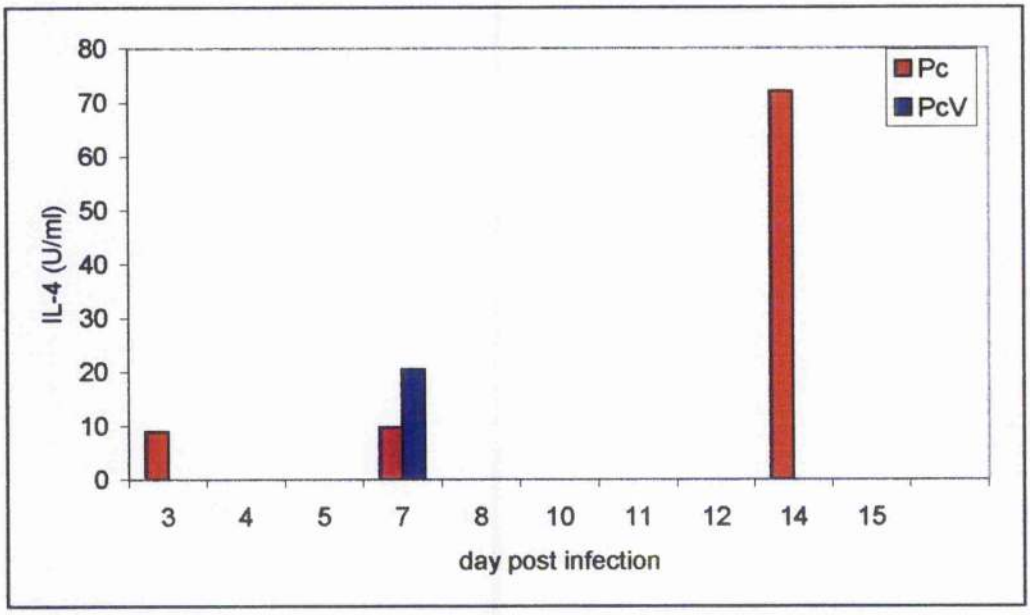


Figure 6.2. IL-4 concentration in lysed whole blood of 10-12 week old male BALB/c mice infected with *P. c* AS. Both groups (Pc and PcV) were infected i.p. with $1x10^5$ pRBC and received 100µl PBS and NISV respectively s.c. on days -28 and -14. Results represent pooled samples of 2 animals per group at each time point indicated.

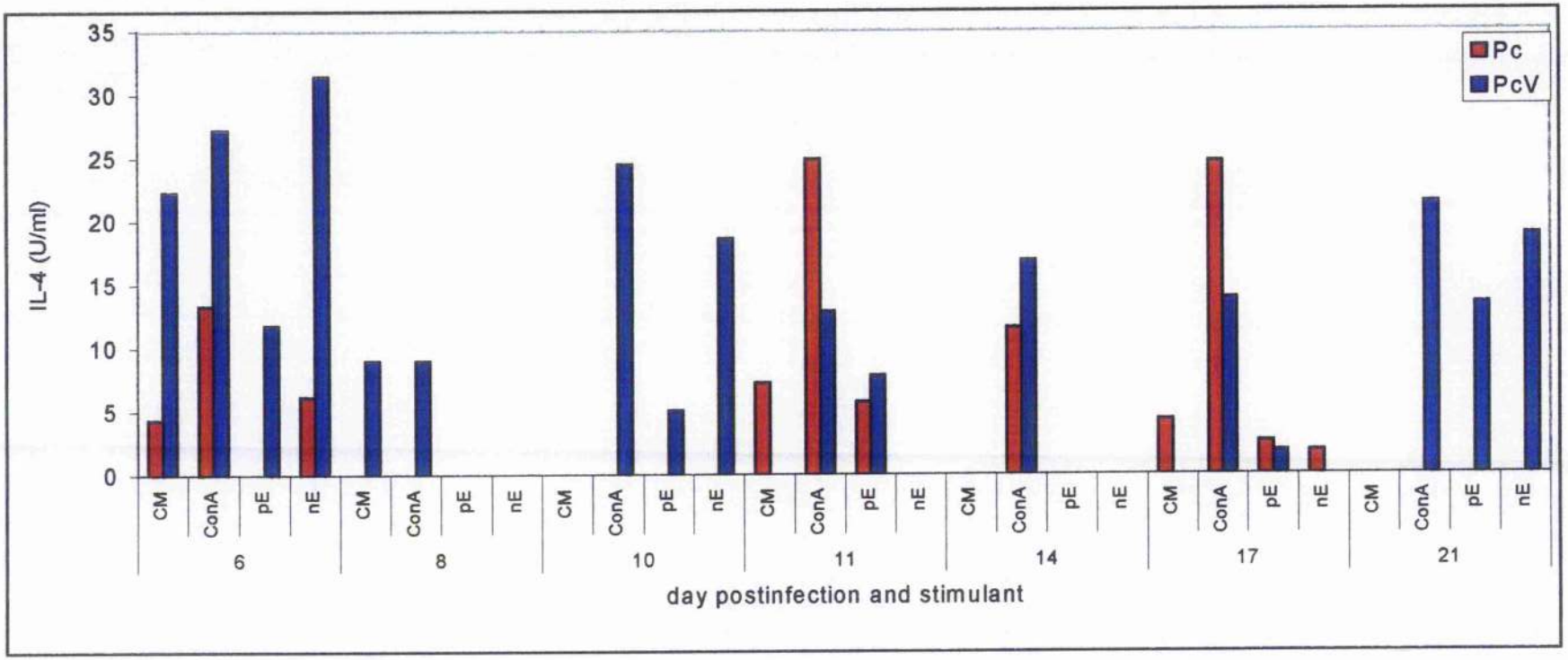


Figure 6.3. IL-4 levels in splenocytes supernatants of 10-12 week old male BALB/c mice with *P. c. c* AS infection. Pc and PcV groups received $1x10^5$ parasitised red blood cells on day 0 and 100µl of PBS or NISV respectively on days –28 and –14. Results represent pooled samples of two animals per group at each time point indicated. CM; complete RPMI medium, ConA; concanavalin A at 10µg/ml, pE; parasitised erythrocytes and nE; normal erythrocytes.

Discussion:

As mentioned earlier, the studies presented in this thesis represent 4 experiments conducted at two venues, Strathclyde University and Glasgow University. IL-4 results presented in this Chapter are from plasma samples of an experiment conducted at Strathclyde University and from lysed whole blood and splenocytes supernatant of an experiment conducted at University of Glasgow i.e. a total of two experiments out of four. The results presented here are discussed in two contexts, the relation between IL-4 levels and disease severity and the effect of pre-treatment with NISV on the IL-4 levels in *P. c.* AS-infected BALB/c mice.

IL-4 levels in the plasma of the mice in the first experiment were not detected until late during infection (day 12), after the peak of parasitaemia which occurred on day 8. In general higher levels of IL-4 were detected in the NISV-treated group (PcV) than the untreated group (Pc) on days 12-15. In this particular experiment the treated group (PcV) was the better one clinically with regards to percent parasitaemia, amount of weight loss and health status score. Higher IL-4 levels in the PcV group, therefore, could be due to pretreatment with NISV or it could merely reflect the disease severity in this group in comparison with the untreated group. Since no plasma samples were available for IL-4 level measurements from more than one experiment, it becomes difficult to attribute the high levels in the PcV group to the NISV. With regards to the relation between IL-4 levels and disease severity, Langhorne and colleagues (1989) have shown a sequential appearance of Th1 cells, with production of IFNγ, and Th2 cells, with production of IL-4, in *P. c.* AS-infected resistant C57Bl/6 mice (Langhorne *et al.*, 1989). Early production of IL-4, on the other hand was found to correlate with susceptibility in *P. c.* AS-infected susceptible A/J mice (Jacobs, Radzioch and Stevenson, 1996). It could be said that the plasma IL-4 levels shown here agree with these studies in the sense that high levels produced late in the infection following peak parasitaemia in the PcV group correlate with resistance to infection since this group exhibited a less severe disease than the Pc group in this experiment. This, therefore, indicates that even within the same mouse species (BALB/c in this case) high IL-4 levels late in the disease could reflect resistance to infection.

Lysed whole blood samples and splenocytes supernatants were obtained from the same animals in a single experiment conducted at Glasgow University. To date I am not aware of whole blood samples being used for measurement of cytokines by ELISA which, therefore, does not allow any comparison to be made with other results. Also these samples were collected from a single experiment and a repeat is needed to confirm the results. It can, however, be seen that high IL-4 levels late in the infection (day 14) in the untreated (Pc) group in the whole blood and in the splenocytes supernatants correlated with protection from infection. On the other hand, high levels in the splenocytes supernatants in the NISV-treated group (PcV) correlated with disease susceptibility since in this experiment, the untreated group exhibited a less severe disease with regards to percent parasitaemia, degree of weight loss and health status score.

Several studies have shown that IL-4 deficiency in IL-4 gene KO mice produces a higher peak parasitaemia and a higher recrudescence but does not impair parasite clearance i. e. does not abrogate immunity (von der Weid *et al.*, 1994, Balmer, 1997). The results presented here also seem to agree with these studies. In both experiments, both Pc and PcV groups whether had a high or a low level of IL-4 at any time during infection, both cleared the parasites and made a full recovery. Also, the two groups had a similar mortality in both experiments.

Collectively the above results indicate that pretreatment of *P. c.* AS-infected male BALB/c mice with NISV does not alter IL-4 levels in their circulation or those produced by their splenocytes. It can also be said that the difference in IL-4 levels between the two groups here merely reflects the difference in their degree of illness and is not due to pretreatment with NISV. Several possibilities have been discussed to explain the difference in the disease severity between the two groups (see Chapter 3).

Chapter 7

Effects of non-ionic surfactant vesicles in *in vitro* setting.

Introduction:

It was seen in Chapter 4 that pretreatment of *P. c.* AS-infected male BALB/c mice with subcutaneous injections of NISV led to suppression of circulating TNFα levels but the clinical outcome was different in different experiments. To increase knowledge about the activity of NISV, their effects *in vitro* was investigated.

Two parameters were chosen initially to investigate the *in vitro* effects of NISV. These were, TNFα production from macrophages and splenocyte proliferation in response to selected stimulating agents in the presence of NISV. Some of the *in vitro* work involved the use of naïve female ICR mice due to shortage of male BALB/c mice from the suppliers.

7. 1. a. Effects of NISV on T cell proliferation *in vitro*:

Results

Splenocytes ($1x10^5$cell/well) from naive 10-12 week old male BALB/c mice were obtained aseptically and stimulated with the mitogen ConA (10μg/ml) in the presence or absence of NISV. Three different concentrations of NISV 2.5, 5 and 10μl/well containing a total volume of 200μl were used to investigate any dose-dependent response to the NISV. Splenocytes were incubated at 37°C in 5% CO_2 in air for 72 hr when they were pulsed with ½ μCi of ^{3}H-methyl-thymidine (Amersham) and incubated for a further 18 hr. Cells were then harvested onto a filter paper and air-dried. The beta activity of each sample was determined by a scintillation counter (see Materials and Methods for details).

Initial results showed that NISV had a suppressive effect on splenocyte proliferation in response to ConA and that the suppression was dose-dependent. See Figure 7.1.
To confirm these preliminary results, this work was repeated but with a different protocol of NISV addition to the cells to avoid handling very small volumes which could stick to the walls of the wells rather than mix with the contents. See Table 7.1.

Table 7.1: Sequence of addition of substances to culture wells. ConA; concanavalin at 10μg/ml, CM; complete RPMI medium.

protocol	Sequence of addition of materials to culture plate wells
0	Splenocytes (100μl) then 100μl of CM or Con A (10μg/ml)
1	splenocytes (100μl), ConA (100μl), NISV (2.5, 5 or 10μl)
2	splenocytes (100μl), [ConA (100μl) +NISV (2.5, 5 or 10μl)] mixed well with Con A
3	splenocytes (100μl), ConA (50 μl of 10μg/ml), NISV(2.5, 5, 10μl made up to 50μl in CM.
4	splenocytes (100μl), NISV (2.5, 5, or 10μl made up to 50μl), ConA (50μl)

Following the above protocols of adding NISV to splenocytes, revealed an as yet unreported property of NISV. It showed that the suppressive effect was dependent on the protocol of NISV addition to the spleen cultures. While addition of small volumes to the wells exerted dose dependent inhibitory effects on the proliferation, using bigger volumes (but same doses) of NISV completely inhibited splenocyte proliferation regardless of the dose and regardless what the NISV were mixed with. This clearly indicated that small volumes of NISV added to the wells did not mix well with the cells and may have formed a film on the surface and hence producing only limited effects while good mixing with the cells produced the full effect (Figure 7.1)

This total suppression of splenocytes by NISV was an unexpected finding even to the suppliers of NISV (Dr. J. Brewer, Division of Immunology, Infection & Inflammation, Glasgow). One hypothesis suggested to explain this observation was that ConA diffused into the NISV and so was no longer available for splenocytes. The experiment was, therefore, repeated with other stimulating agents namely lipopolysaccharide (LPS), pRBC and nRBC in addition to complete medium. It was again found that NISV mixed in relatively large volumes, totally suppressed splenocytes proliferation in response to any of the above stimulants regardless of the NISV dose (Figure 7.2). The hypothesis that ConA diffused into NISV and was,

therefore, not available to stimulate the splenocytes was rejected because proliferation was suppressed in response to all stimulants used, some of which, such as erythrocytes, are in fact larger than the vesicles (7-8μm v. 255nm).

In order to investigate the mechanisms of this suppression, splenocytes were exposed to NISV for different lengths of time (1 and 3 hr) and then centrifuged in a hope that a lipid film containing NISV would form on the surface. The plan was to remove this film and expose the cells to ConA or complete medium to see if the time of exposure to NISV had any effect on the proliferative response of splenocytes to ConA. Contrary to the belief of the suppliers (Dr. J. Brewer, Division of Immunology, Infection & Inflammation, Glasgow), this expected film did not form no matter what speed of centrifuge was used. In order to remove NISV from the cells, cells were first washed three times in big volumes (30 ml) of complete medium and were then further incubated for 72 hr in the presence or absence of ConA. It was seen that although 1 hr incubation of splenocytes with NISV was not sufficient to suppress their proliferation in response to ConA, 3 hr incubation was sufficient. It was also seen that 1 hr incubation with NISV led to a greater response to ConA than cells not exposed to NISV. See Figure 7.3.

One hypothesis suggested by the suppliers of NISV (Brewer and Alexander, personal communication) was that the NISV were toxic to the splenocytes due to the detergent effects of their components (cholestrol, dicetylphosphate and monopalmitoly glycol). To investigate this hypothesis, cells viability was checked with trypan blue exclusion method and by MTT assay. At the same time splenocyte proliferation was examined with the ^{3}H uptake. Cell viability studies showed that the exposed cells were in fact viable (Figure 7.4). This clearly indicated that in the presence of NISV, splenocytes remained viable but non-proliferating in response to ConA.

In view of the above results, it was decided to investigate NISV effects on splenocytes in *ex-vivo* settings.

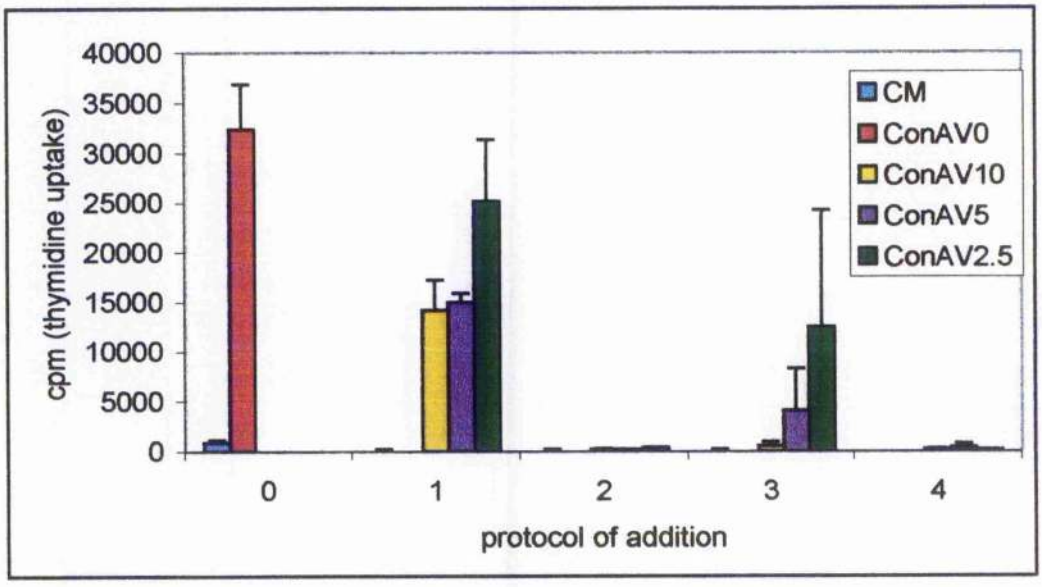


Figure 7.1: Effect of different methods of addition of NISV to splenocytes in culture on proliferation of splenocytes in response to ConA. Splenocytes obtained aseptically from a naive male BALB/c mouse, were stimulated with 10μg/ml of ConA. Three concentrations of NISV were used, 2.5, 5 and 10μl per 200μl well. Results represent means of three wells and error bars are the standard deviations. Details on the protocols are in Table 7.1. ConAV0; [ConA (1μg) + NISV (0μl)] / 200μl, ConAV10; [ConA (1μg) + NISV (10μl)] / 200μl, ConAV5; [ConA (1μg) + NISV (5μl)] / 200μl and ConAV2.5: [ConA(1μg) + NISV (2.5μl)] / 200μl.

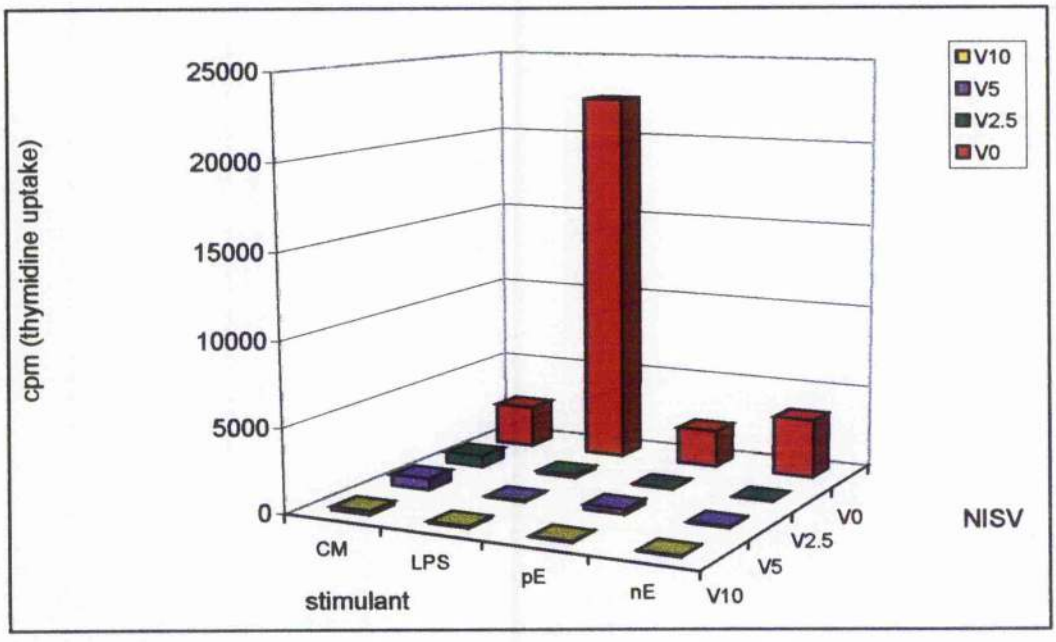


Fig 7.2. NISV effects on splenocytes obtained from a naïve male BALB/c mouse. NISV (2.5, 5 or 10μl/well) were mixed with the stimulating agent to a final volume of 100μl. CM; complete RPMI medium, LPS; lipopolysaccharide (3μg/ml), pE; parasitized mouse red blood cells, nE; normal mouse red blood cells. V0; no NISV, V2.5; NISV (2.5μl/well), V5; NISV (5μl/well), V10; NISV 10μl/well).

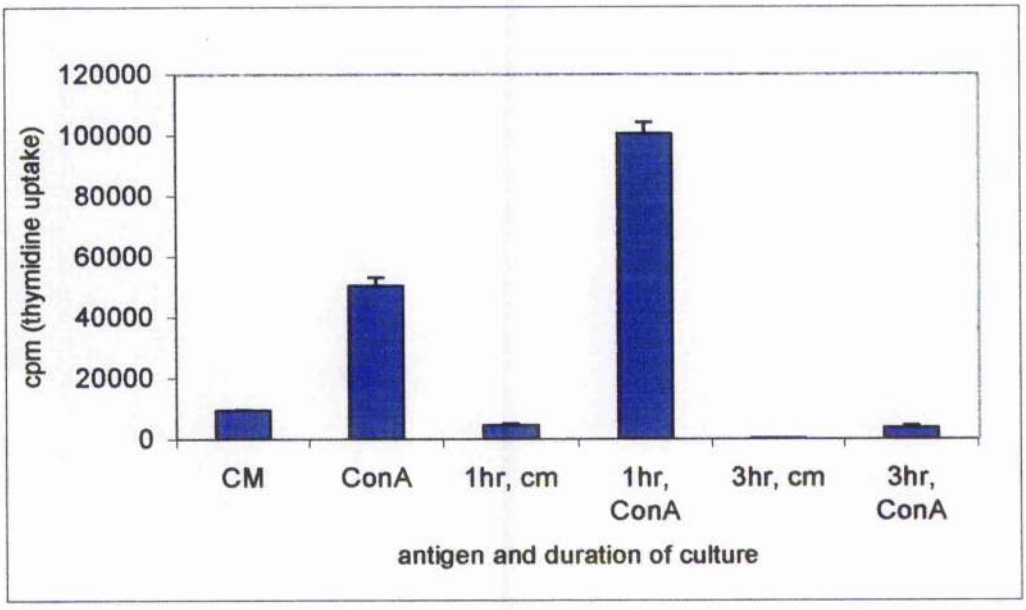


Figure 7.3: Splenocyte proliferation in response to ConA after prior incubation with NISV. Splenocytes were incubated with NISV for 1 or 3 hr. NISV dose chosen here was 5µl/well. CM; complete RPMI medium, ConA; concanavalin (10µg/ml). Results represent means of 3 wells and the bars are the standard errors of means.

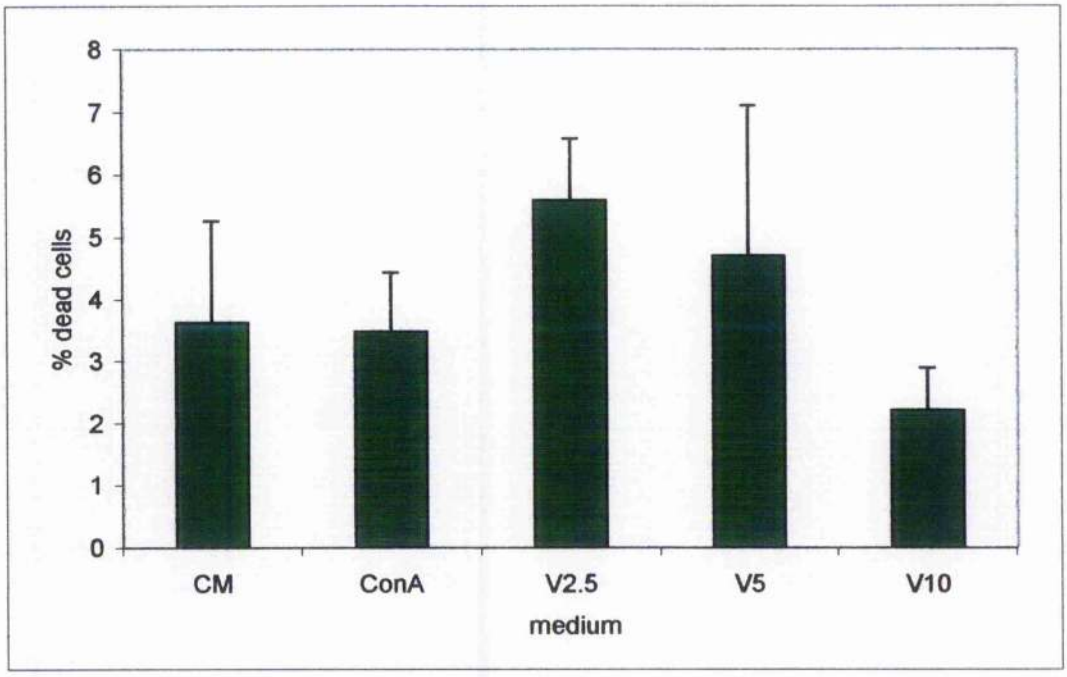


Figure 7.4: Splenocyte viability checked with trypan blue exclusion method. Splenocytes were exposed to 3 concentrations of NISV (2.5, 5 and 10µl/well) mixed with ConA (10µg/ml) to a final volume of 100µl. CM; complete RPMI medium, ConA; concanavalin (10µg/ml), V2.5, V5 and V10 (NISV 2,5, 5 and 10µl/ml respectively). Results represent means of 3 wells and bars are standard deviations

7.1.b. Effects of NISV on proliferation of splenocytes from *P.c.* AS-infected male BALB/c mice:

Results

The results shown here are the combination of two experiments with similar clinical outcome. In both experiments, which were conducted at Glasgow University, the NISV-treated group (PcV) developed a more severe disease than the untreated group (Pc) in terms of level of parasitaemia, degree of weight loss and health status score (see Appendix 2). Twenty and twenty-five male BALB/c mice per group in experiments G.1 and G.2 respectively were infected with $1x10^5$ pRBC on day 0. Groups Pc and PcV were inoculated subcutaneously with 100µl of PBS or NISV on days –28 and –14. Spleens of two animals per group were obtained aseptically at selected time points (days 1, 4, 7, 10 and 16 and days 6, 8, 11, 13, 14, 17 and 21 post infection in experiments G.1 and G.2 respectively). Splenocytes from the PBS and NISV groups were obtained on all time points of experiment G.1 and on day 21 only of experiment G.2. Splenocytes from each pair of mice were pooled and exposed to various stimulating or control agents ConA (10µg/ml), parasitized erythrocytes, uninfected mouse erythrocytes and complete RPMI medium. After 72 hrs, cells were pulsed with ^{3}H-methyl-thymidine (Amersham) and further incubated for 18hr. Splenocytes were then harvested on a filter paper and the beta activity was read using a scintillation counter (see Material and Methods for details).

The combined *ex-vivo* studies on splenocyte proliferation revealed several points. They showed that, in general, splenocytes proliferative response is greatest to ConA. The two infected groups, Pc and PcV, had a greater response than the control groups on day 1 post-infection only, while a lower response than the control groups was seen at all other time points. The PcV group showed a slight and non-significant lower response than the Pc group at all time points except day 10 when the PcV response was slightly higher. A depressed response of the Pc and PcV groups is seen on days 10-16 post-infection. No difference was seen between the PBS and NISV. An interesting point is that the two control groups PBS and NISV showed a low response on day 1. It can be seen that NISV did not completely suppress splenocytes

proliferation at any time point (Figure 7.5). Results of day 21 post-infection (experiment G.2) are not included due to contamination of the culture with a fungal growth. In the two control groups, PBS and NISV, a variablility in splenocyte proliferation response to ConA was seen at different time points. This variability indicates that the proliferation responses of splenocytes in different media could not be compared with each other at different time points but rather at each single time point.

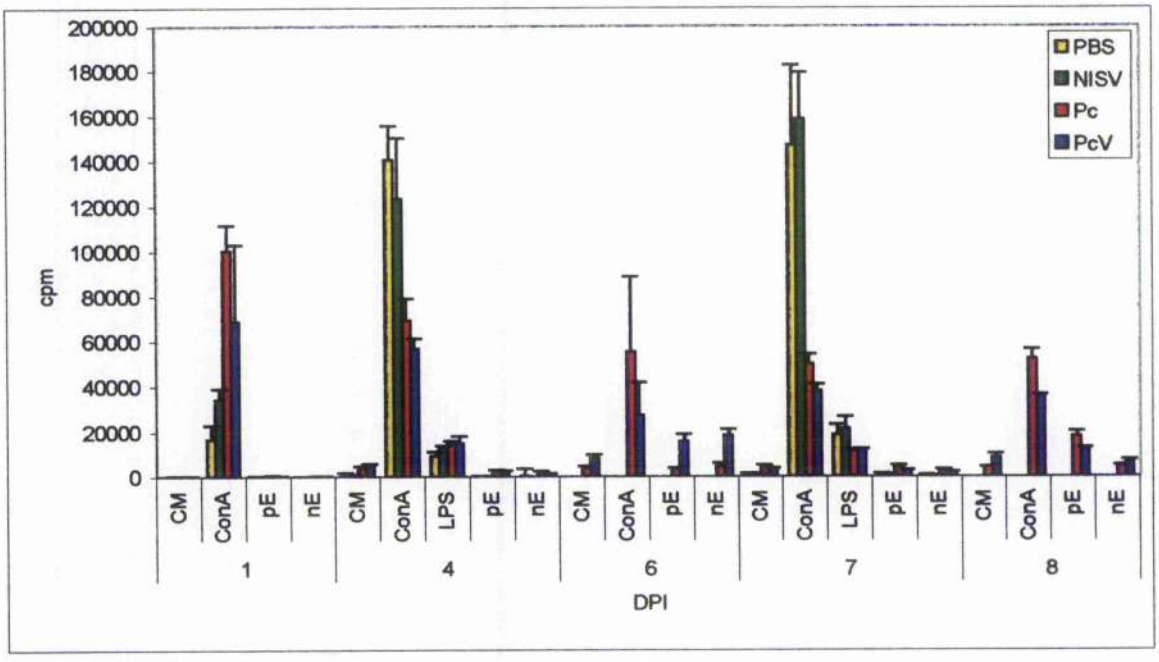


Figure7. 5. a

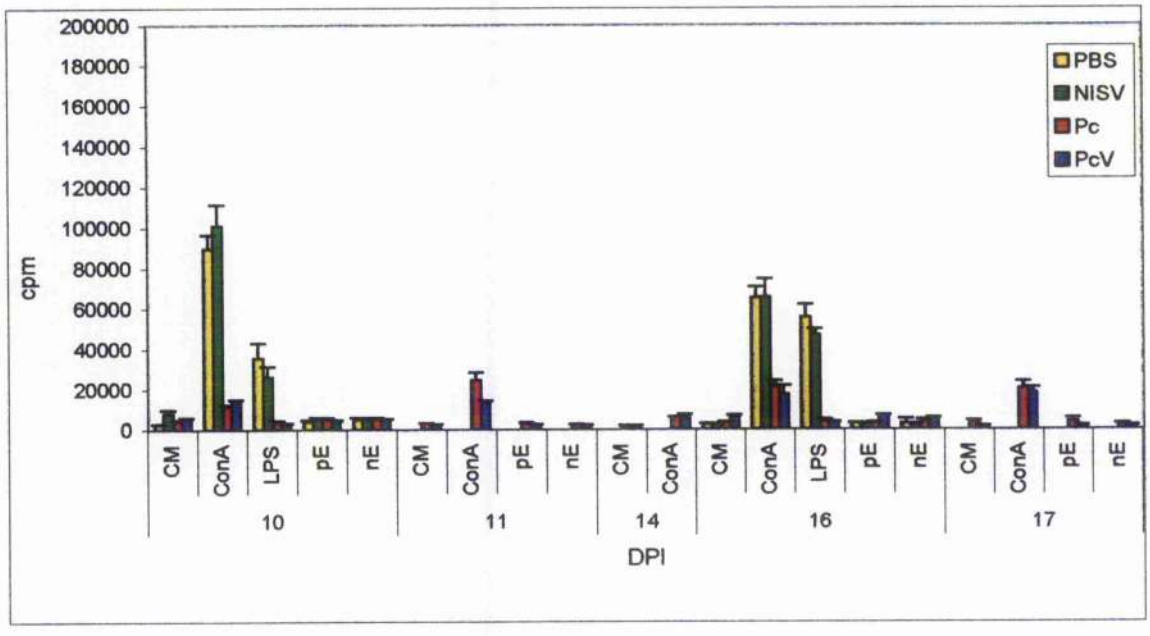


Figure 7. 5. b

Figure 7.5. a, b: Splenocyte proliferation of male BALB/c mice infected with *P. c* AS. Pc and PcV groups were infected with $1x10^5$ parasitized red blood cells on day 0 and inoculated subcutaneously with 100µl of PBS or NISV respectively on days –28 and –14. Groups PBS and NISV were injected subcutaneously with 100µl of PBS or NISV respectively on days –28 and –14 but were not infected. CM; complete RPMI medium, ConA; concanavalin A(10µg/ml), LPS; lipopolysaccharide (3µg/ml), pE; parasitized red blood cells and nE; normal red blood cells. Results represent means of three wells of pooled splenocytes and the bars are the standard deviations.

The next step was to investigate the mechanisms of NISV-induced splenocyte suppression *in vitro*. Several agents are known to suppress T cell proliferation *in vitro* such as nitric oxide (Ahvazi *et al.*, 1995) and PGE2 (Riley *et al.*, 1989). It was, therefore, decided to measure these chemicals in the supernatants of splenocytes exposed to NISV. Nitric oxide levels were measured first and are presented here.

7.2.a. Effects of NISV on the production of nitric oxide from macrophages *in vitro:*

Nitric oxide in a biological setting was first described in 1987 when it was recognized to be the biological agent known to relax endothelium smooth muscle named endothelium derived relaxing factor (EDRF) (Palmer *et al.*, 1987).

Nitric oxide is produced from the terminal guanidine nitrogen of the amino acid L-arginine during its conversion to L-citrulline, a reaction catalysed by the enzyme nitric oxide synthase. Three major forms of this enzyme have been identified. The neural and endothelial forms (Dinerman *et al.*, 1994) are expressed constitutively, produce limited amounts of NO and are calcium and calmodullin-dependent (Bredt and Snyder, 1990). The macrophage type is not normally expressed but can be induced by a variety of factors and hence is known as inducible NO synthase (iNOS). The inducible enzyme produces greater amounts of NO and is Ca-independent (reviewed by Clark and Rockett 1996). Induction of iNOS requires gene transcription which occurs rapidly in response to various stimuli including TNFα, IFNγ, TNFβ, IL-1 (Rockett *et al.*, 1992). It is down regulated by IL-4, IL-5, IL-8, IL-10 and glucocorticoids (reviewed by Cox and Liew, 1992).

Nitric oxide itself cannot be measured because it undergoes rapid oxidation in aqueous solutions to form nitrite and nitrate and it is these products that are measured in blood or urine. The rapid oxidation of NO is due to its chemical nature. NO is a radical molecule with a free unpaired electron in its outer orbit, making it highly unstable and so it reacts rapidly with the surrounding radicals such as superoxide to form highly reactive derivatives such as peroxynitrite ion which is highly toxic to

many molecules including nucleic acid, lipids and proteins (van der Vliet *et al.*, 1994).

Nitric oxide is produced by a variety of cell types including neurons, vascular endothelial cells (Oswald *et al.*, 1994), platelets, neutrophils (McCall *et al.*, 1989), adrenal cells, respiratory epithelial cells, activated macrophages (Stuehr *et al.*, 1985), hepatocytes (Nüssler *et al.*, 1991) and mast cells.

NO is involved in a wide range of biological functions. For example it acts as a neurotransmitter as is thought to be in the case of non-adrenergic, non-cholinergic bronchodilating neurons and it has been used in the treatment of adult respiratory distress syndrome. It is thought to be a major controller of cerebrovascular tone (Wei *et al.*, 1993). Moreover, it is thought to be the principal cytotoxic mediator of effector molecules involved in intracellular parasite killing (Rockett *et al.*, 1991). NO inhibits iron-sulphur-dependent enzymes involved in cellular respiration, energy production and reproduction (Hibbs *et al.*, 1988). Also, NO_2^- synthesis has been shown to correlate directly with intracellular killing of *Leishmania major* (Green *et al.*, 1990).

In the case of malaria, although a saturated solution of NO did not inhibit *P. falciparum* growth *in vitro*, its downstream products, nitrite and nitrate ions, however, were found to be toxic in millimolar concentrations (Rockett *et al.*, 1991). *In vivo* studies also indicated a role for NO in protection against malaria and emphasized that the site and time of NO production were important. *Plasmodium chabaudi*-infected resistant and susceptible mice were found to produce high levels of iNOS in their spleens early during infection and in the liver late in infection respectively (Jacobs, Radzioch and Stevenson, 1996). This follows the same pattern as TNFα production by susceptible and resistant mice infected with *P. c.* AS (Jacobs, Radzioch and Stevenson, 1996) indicating that NO could be the downstream mediator of TNFα–induced resistance. This was confirmed in *P. c* AS-infected mice were treated with anti-IFNγ and anti-TNFα monoclonal antibodies. These mice had a reduced splenic iNOS mRNA production and reduced circulating NO_3^- levels by 50% in mice treated with anti-IFNγ antibodies and 100% in those treated with anti-TNFα antibodies (Jacobs, Radzioch and Stevenson, 1996). $CD4^+$ Th1 cells were found to be the major

source of NO during *P.c.* AS blood stage infection. When mice depleted of $CD4^+$ cells and reconstituted with Th1 cells were treated with L-N^G-monomethyl arginine (L-NMMA), they produced little or no detectable nitrates and developed a greater blood parasitaemia (Taylor-Robinson and Phillips, 1994). It is important to note, however, that NO was found not to be essential in protection against malaria. Treatment of mice with L-NMMA and hydroxybutyrate did not significantly increase parasitaemia or alter the course of the disease in *P. vinkei and P. c. adami* infections (Clark *et al.*, 1987). The authors suggested that this was due to the rapid scavenging of NO by the surrounding haemoglobin. This was in agreement with previous study by Jones and colleagues. TO mice infected with *P. berghei* of *P. vinkei peteri,* lung, liver, kidney and spleen iNOS activity remained unchanged in the lethal and non-lethal infections (Jones *et al.*, 1996).

Nitric oxide, however, was also reported to play a role in the pathogenesis of experimental cerebral malaria (ECM). TNFα/β double KO mice infected with *P. berghei* ANKA were protected against CM. NO production was impaired in these mice suggesting that TNFα-induced NO had a role in CM pathology (Rudin *et al.*, 1997). Other studies, however, found no correlation between NO and ECM. Treatment of *P. berghei* ANKA-infected CBA mice with L-NMMA did not prevent the development of ECM (Kremsner *et al.*, 1993). Also, it was found that *P. c* AS-infected C57Bl/6 mice deficient in iNOS and given aminoguanidine to block other NOS ran a similar course of infection to wild mice with no difference in the parasitaemia course, survival and haematological values (Favre, Ryffel and Rudin, 1999).

In human *P. falciparum* malaria, some studies reported a protective role of NO. Leukocyte NOS2 was not detected in children with CM, while in all those with subclinical infection and in controls, NOS2 was detectable. The authors suggested that NO synthesis protected against clinical disease (Anesty *et al.*, 1996). Other studies, however, reported a pathological role for NO in CM. Serum levels of reactive nitrogen intermediates were found to correlate positively with the depth of coma in children with CM at admission and its duration (Al-Yaman *et al,* 1996).

Apart form its effects on the blood stage malaria, NO was also found to play a part in the immune response mounted against the intra-hepatic forms of the malaria parasite. L-NMMA was found to block IFNγ-induced inhibition of sporozoite growth *in vitro* indicating that the inhibition was mediated through NO (Mellouk *et al.*, 1991). NO was found to promote the accumulation of $CD4^+$ and $CD8^+$ T cells round infected hepatocytes in *P. berghei* infected Brown Norway rats (Scheller *et al.*, 1997).

Moreover, NO was shown to reduce gametocyte infectivity in both rodent (Motard *et al.*, 1993) and human malaria (Naotunne *et al.*, 1993). Gametocyte infectivity to *A. stephensi* was reduced when the mosquito feeding occurred during time of schizogony in the *P. vinkei peteri*-infected mice. This, however could be prevented by treatment of the mice with L-arginine analogue Nw-nitro-L-arginine (NwNLA) prior to mosquito feeding, indicating that the suppression of the gametocyte infectivity was due to nitrogen reactive intermediates (Motard *et al.*, 1993).

On the other hand, NO has been reported to be responsible at least in part for immunosuppression (Abvazi *et al.*, 1995) which occurs during acute malaria infection (Rockett *et al.*, 1994). It has been reported that lymphocytes from malarial mice develop a poor proliferative response to ConA (Corrêa *et al.*, 1980) and foreign red blood cell antigens (Greenwood *et al.*, 1971). It is this last property, which is of interest to the work done here.

Results:

Splenocytes cells were obtained aseptically from naïve 10-12 week old female ICR mice. $1x10^5$ cells per well were stimulated with LPS (3µg/ml) and NISV was added to the cells in three different concentrations (2.5, 5 and 10µl/well). Samples were run in triplicates with each well containing a final volume of 200µl. Cells were incubated for 72 hrs at 37°C in 5 % CO_2 in air and the supernatants were collected and stored at -20°C until further analysis. The same procedure was applied to adherent murine hepatic and bone marrow (BM) cells. Production of NO was measured as nitrates by the Greiss reaction (for details see Materials and Methods).

It was found that LPS did not stimulate NO production from splenocytes and liver adherent cells more than that produced in the presence of complete RPMI medium. LPS, however, did induce NO production by BM adherent cells. NO production was enhanced by NISV in a dose-dependent manner from all three cell types (see Figure 7.6 a and b). In view of these results, it was decided to investigate the effect of L-NMMA on the reversal of NISV-induced suppression of splenocytes proliferation and whether NO was the only agent involved or others were also involved. Initially only one L-NMMA dose (500μM/well) was used and it showed that there was some recovery of splenocyte proliferation of cells exposed to 2.5μl of NISV/well (see Figure 7.7).

In view of the above results, it was decided to investigate the effects of NISV treatment of splenocyte NO production in *ex- vivo*.

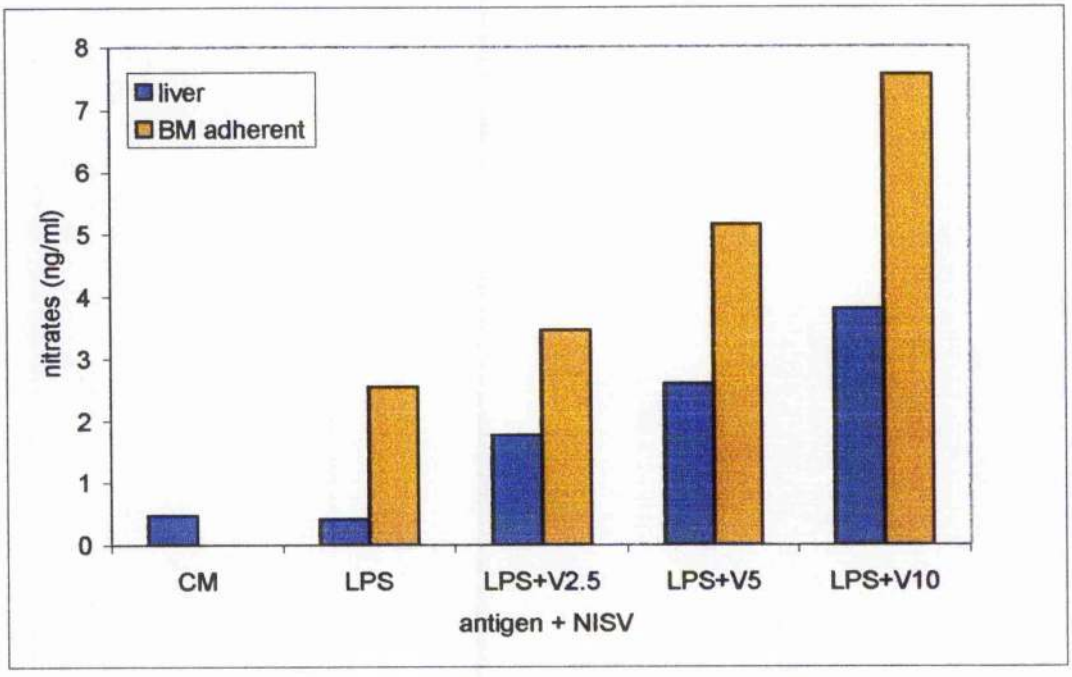


Figure 7.6 a. Nitric oxide production from adherent liver and bone marrow cells. Cells were removed aseptically from naive female ICR mice and stimulated with LPS in the absence or presence of three different concentrations of NISV (2.5, 5 and 10μl/well). CM; complete RPMI medium, LPS; lipopolysaccharide (3μg/ml), V2.5; NISV (2.5μl/well), V5; NISV (5μl/well), V10; NISV (10μl/well). Results represent means of 3 wells.

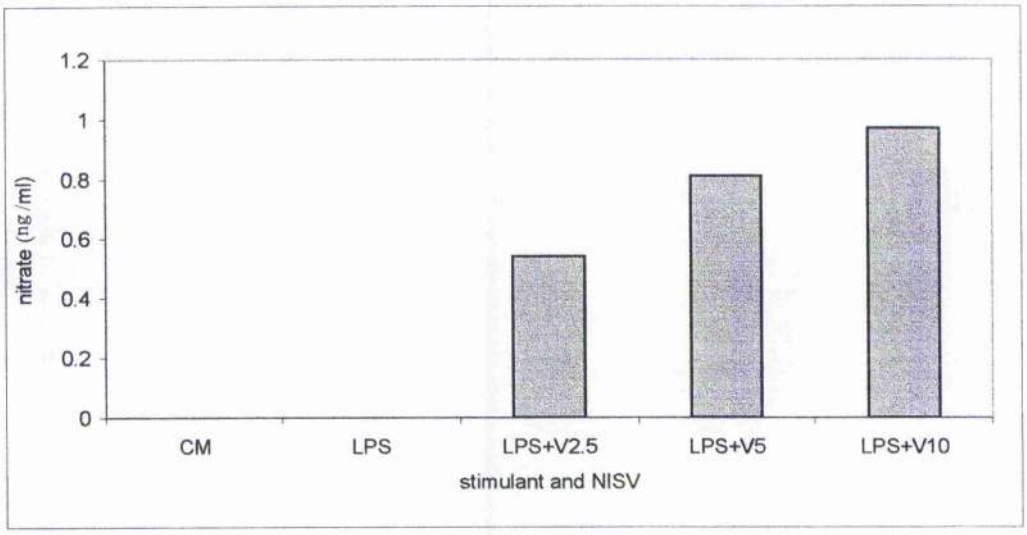


Figure 7.6 b. NO production from splenocytes in response to LPS and NISV. CM; complete RPMI medium, LPS; lipopolysaccharide (3μg/ml), V2.5; NISV at 2.5 μl/well, V5; NISV at 5μl/well and V10; NISV at 10μl/well. Results represent means of three wells. Note that NISV induces production of nitrates by splenocytes in a dose-dependent manner.

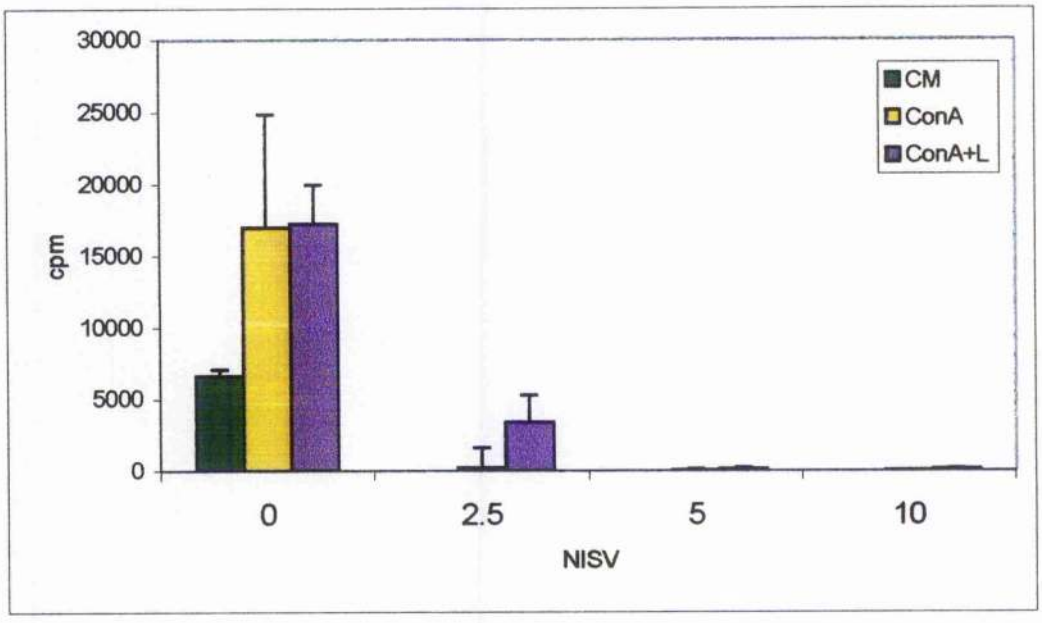


Figure 7.7: Splenocyte proliferation in response to ConA (10μg/ml) and presence of NISV (2.5, 5 or 10μl/well) and LNMMA (500μM/well). Spleens were obtained from a naïve female ICR mouse. Results represent means of three wells and bars are standard deviation. CM; complete RPMI medium, ConA; concanavalin (10μg.ml), L; L-NMMA (500μM/well). Note that there was some recovery of proliferation by cells exposed to 2.5μl/well of NISV.

7.2.b. Effects of NISV treatment on the *ex-vivo* production of NO from splenocytes:

Results:

Twenty five male BALB/c mice per group were infected with $1x10^5$ pRBC on day 0. Groups Pc and PcV were treated subcutaneously with 100µl of PBS or NISV respectively on days –28 and –14. Spleens of two mice per group were removed aseptically at selected time points (days 6, 8, 11, 14, 17 and 21 post-infection) during infection and pooled. Spleens were obtained from the PBS and NISV groups on day 21 post-infection only. Splenocytes were then exposed to various stimulating agents including; ConA, pRBC, nRBC plus complete RPMI medium and incubated at 37°C in 5% CO_2 in air for 72 hrs, at which time supernatants were collected and stored at –20°C until further analysis. Nitrate was then measured by the Greiss reaction in samples run in duplicates.

The NISV-treated group produced less NO than the untreated group on days 14 and 17 in response to ConA. On the other hand, NO production was higher in the treated group (PcV) on days 6 in response to ConA, parasitized and non-parasitized erythrocytes. It was also found that both the Pc and PcV groups produced high amounts of NO on day 21 post-infection in response to all stimulants even complete RPMI medium. No difference was seen between the PBS and NISV groups (Figure 7.8).

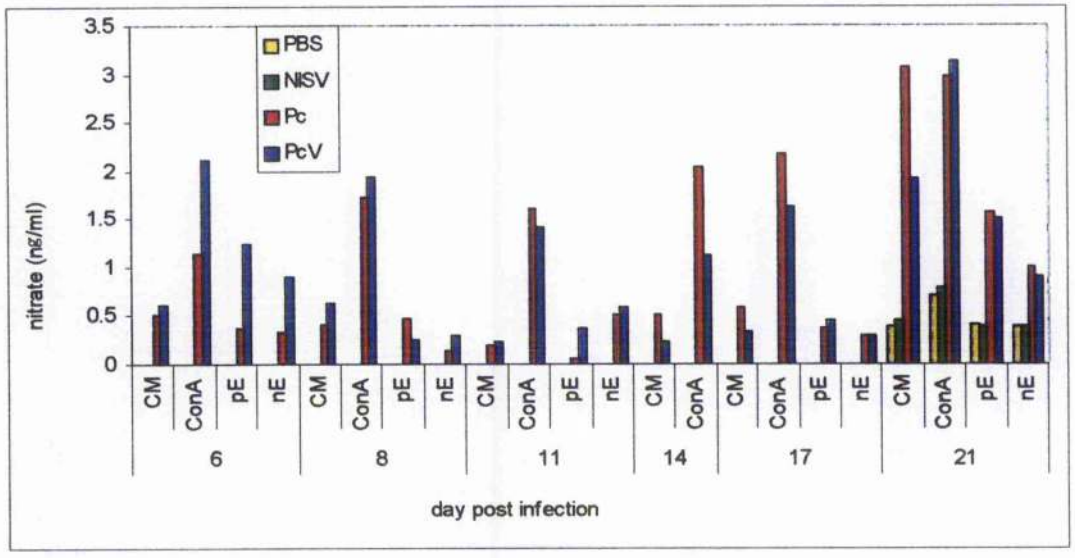


Figure 7.8: NO production by splenocytes of male BALB/c mice infected with *P.c.* AS. 25 male BALB/c mice per group were infected with $1x10^5$ pRBC on day 0. Groups Pc and PcV were given 100µl of PBS or NISV respectively on days –28 and –14 via subcutaneous inoculation. CM; complete RPMI medium, ConA; concanavalin (10µg/ml), pE; parasitesed red blood cells, nE; normal erythrocytes.

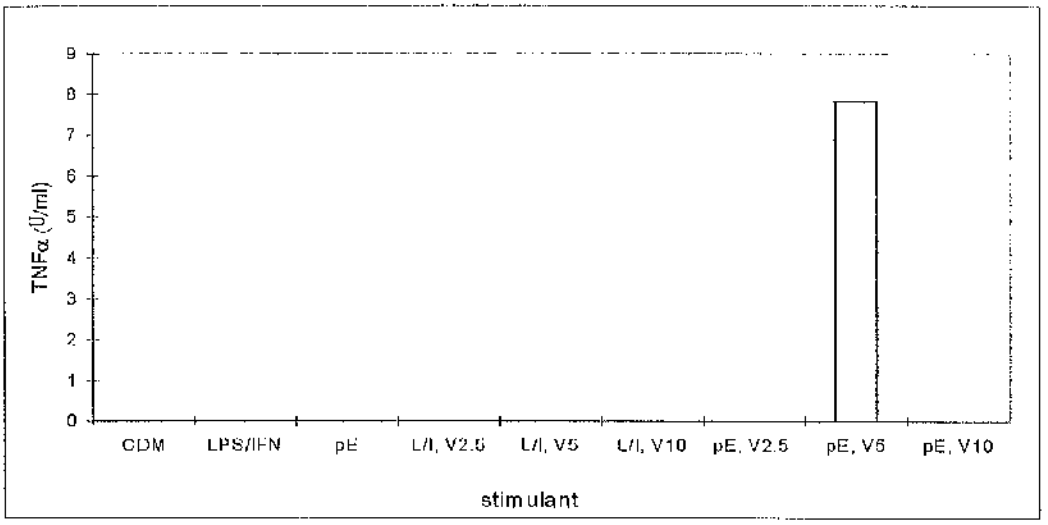
7. 3. Effects of NISV on TNFα production *in vitro*:

As seen in Chapter 4, pretreatment of male BALB/c mice with NISV led to suppression of circulating TNFα but with a variable clinical outcome in different experiments. Also previous work with the vesicles revealed that they suppressed TNFα production *in vitro* from murine bone marrow derived macrophages and human peripheral blood lymphocytes exposed to LPS (Roberts, Brewer and Alexander patent application, 1995). One of the aims of this project was to confirm the suppressive effects of NISV on TNFα production *in vitro*.

Results:

Spleens of naive 10-12 week old male BALB/c mice were removed aseptically. Splenocytes ($1x10^5$ cells/well) were incubated with LPS (3μg/ml) for various lengths of time (2, 4, 6, 18, 24 and 48hr). Supernatants were then collected and stored at –20° C until further analysis. Initially no TNFα was detected at any time point chosen and so cells were exposed to a combination of LPS (250ng/ml) and IFNγ (100U/ml). Again TNFα was not detected and the LPS was thought to have expired. The above work was repeated with using fresh LPS but still without success. It was then decided to obtain macrophages from the bone marrow and investigate NISV effects on TNFα production. This seemed very reasonable since previous work by the NISV suppliers (Dr. J. Brewer, Division of Immunology, Infection & Inflammation, Western Infirmary, Glasgow) involved the use of bone marrow derived macrophages (Roberts, Brewer and Alexander, 1995 patent application). Bone marrow of 10-12 week old female naive ICR mouse was obtained aseptically and cells were allowed to grow for a week in DMEM supplemented with 20% FCS and 30% supernatants of L-929 cells in 5% CO_2 in air (Wolfarm *et al.*, 1996). Non-adherent cells were then removed and the adherent cells were collected, washed twice and stimulated with LPS (3μg/ml), LPS (250ng/ml) + IFNγ (100U/ml) or complete DMEM medium in the presence of NISV (2.5, 5, 10μl/well). Cells were incubated for various lengths of time and supernatants collected and stored at –20°C until further use. Hepatic adherent cells were also exposed to the above agents and NISV.

With regards to liver and bone marrow adherent cells, here too TNFα proved to be difficult to detect and experiments had to be repeated several times. TNFα was never detected in supernatants of cells stimulated with LPS or a combination of LPS and IFNγ. TNFα was never seen to be suppressed by NISV in any experiment, in fact in some, it was seen to induce its production. TNFα was detected in supernatants of BM-derived macrophages stimulated with parasitized erythrocytes in presence of 5µl/well of NISV at two hr incubation. At six hr incubation, TNFα was detected in the supernatants of BM-derived macrophages stimulated with LPS + IFNγ and with parasitized red blood cells in the presence of 10µl/well of NISV (Figures 7.9 a and b). TNFα was also detected in the supernatants of hepatocytes incubated with LPS + IFNγ and NISV 5µl/well for two hrs (Figure 7.10).



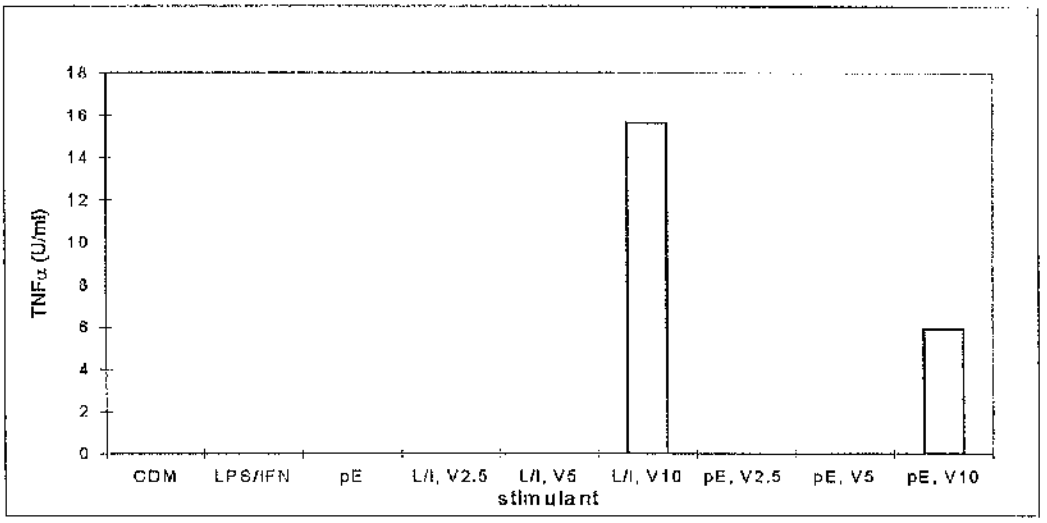


Figure 7.9 a and b: TNFα production by bone marrow macrophages of female naïve ICR mouse. Cells were stimulated by LPS (250ng/ml) and IFNγ (100U/ml) or parasitized red blood cells in the presence of 3 doses of NISV(2.5, 5, and 10µl/well). Supernatants were collected at 2, 4, and 6 hr. CDM; complete DMEM medium, L/I; LPS and IFNγ, pE; parasitized erythrocytes, V2.5; NISV (2.5µl/well), V5; NISV (5µl/well), V10; NISV (10µl/well).

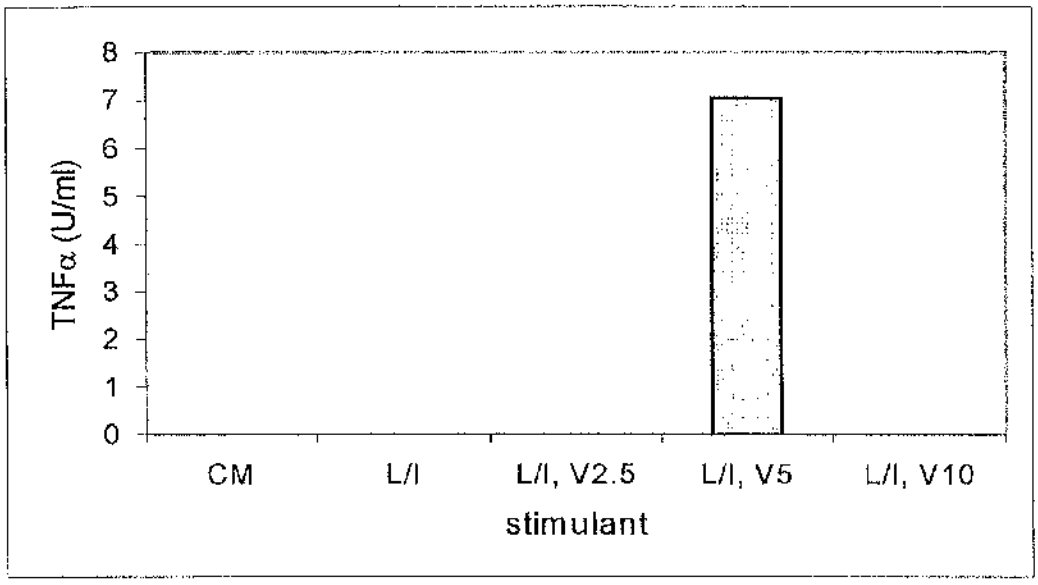


Figure 7.10: TNFα production from liver adherent cells obtained from female ICR mouse and stimulated with LPS and IFNγ. Cells were exposed to 3 different doses of NISV (2.5, 5 and 10μl/well) and supernatants were collected at 2 and 4 hr intervals. CM; complete RPMI medium, L/I; LPS (250ng/ml) and IFNγ (100U/ml), V2.5; NISV (2.5μl/well), V5; NISV (5μl/well), V10; NISV (10μl/well).

7. 4. Comparison between different samples in measuring cytokines:

As mentioned before, difficulty was faced in measuring TNFα by the ELISA method. Results presented in this thesis were achieved after several unsuccessful attempts. In view of the difficulty of measuring TNFα, it was decided to measure cytokine levels in different samples (serum, plasma and whole blood). It was also decided to investigate the effects of repeated freezing and thawing of the samples on the levels of cytokines detected.

Results:

Seven female ICR mice (age 12-14weeks) were infected with $1x10^5$ parasitized red blood cells on day 0. Two animals were sacrificed on days 4, 8 and 10 post infection and blood was collected via cardiac puncture. Blood was divided equally into 3 Eppendorfs with one Eppendorf containing heparin (100µl/ml). The aim was to have plasma, serum and whole blood. To obtain plasma, blood was centrifuged at 13000 rpm (11,600g) for 3 min, plasma removed and stored at –20°C until further use. Serum was obtained by leaving blood at room temperature overnight to coagulate. Serum was then collected and stored at –20°C until further use. Since whole blood did not contain any anticoagulant, it clotted. In order to keep the whole blood samples in a liquid state to allow their use in ELISA, a few drops of ddH_2O were added to whole blood before freezing it (100µl/1ml of blood). Whole blood samples (containing ddH_2O) were than stored at –20°C until further analysis. Packed blood cells remaining following the removal of were also liquidized by addition of ddH_2O and stored at –20°C.

As mentioned previously, steps in the ELISAs were modified when working with whole blood (see Materials and Methods). Following 1 hr incubation of whole blood samples with the coating monoclonal antibody, plates were kept at 37°C over night and washed the following day. The rest of the steps followed the usual ELISA method steps.

With regards to TNFα, it can be seen that no TNFα was detected in the plasma samples at any time points while some TNFα was detected in the serum samples on days 8 and 10 post infection and in whole blood at all the 3 time points. Highest levels of TNFα, however, were detected in the packed cells remaining after the removal of plasma, followed by packed blood cells remaining after the removal of serum. On day 8 post infection, no samples were available for packed blood cells remaining following the removal of plasma or serum (Eppendorfs were discarded by mistake). In this preliminary experiment, control samples were obtained from a PBS treated male BALB/c mouse from the previous experiments. Note that there is a difference in the levels detected in whole blood haemolysed with ddH_2O and that without it. No TNFα was detected in the control group. See Figure 7.11.

IFNγ was measured repeatedly in the above samples following second, third and sixth time thawing of the samples. Levels shown here were detected after the second and sixth thawing of the samples. IFNγ was not detected in either plasma or serum samples at any time point. No IFNγ was detected in blood with or without water after the second thawing of the samples at any time point. After the sixth thawing, however, similar levels of IFNγ were detected in blood with and without water (no sample for blood without water was available on day 10 post-infection). Similar levels of IFNγ were detected in the whole blood with ddH_2O and whole blood without ddH_2O on days 4 and 8 post-infection after the sixth time thawing of the samples (no samples of whole blood without ddH2O were left on day 10 after the sixth thawing). IFNγ level in packed cell remaining after removal of plasma (Bp) on day 4 was approximately 200 U/ml following the second and the sixth time thawing of the samples, while no IFNγ was detected in packed cells remaining after the removal of serum (Bs) at this time point. Furthermore, IFNγ levels in Bs on day 10 post-infection were <50U/ml after the second thawing while levels >140U/ml were detected after the sixth time thawing of the sample. Approximately 200U/ml of IFNγ were detected in Bp on day 10 after second and sixth thawing of the sample (Figures 12.7. a and b).

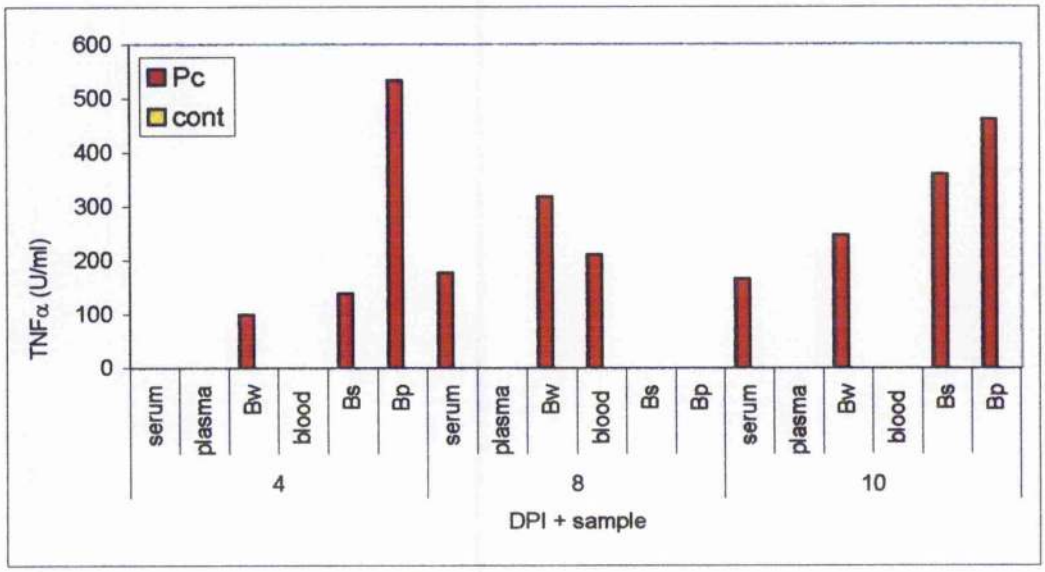
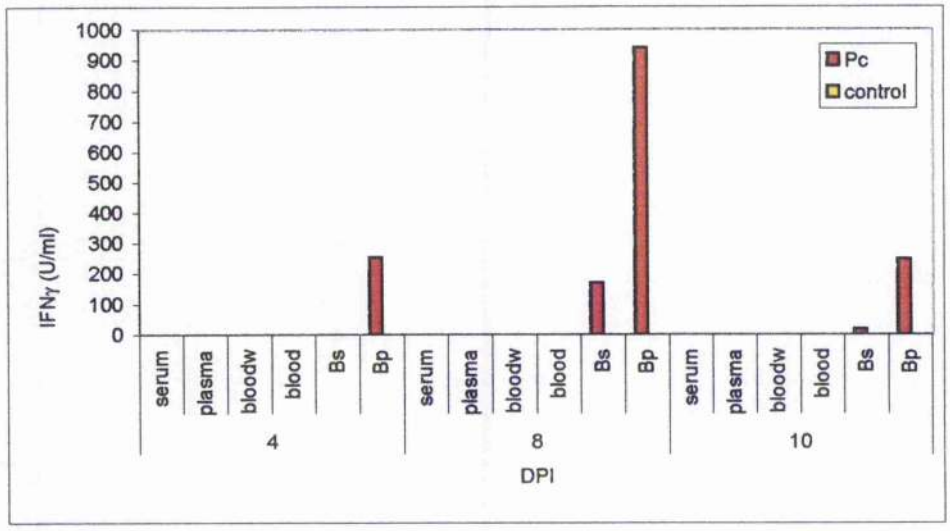
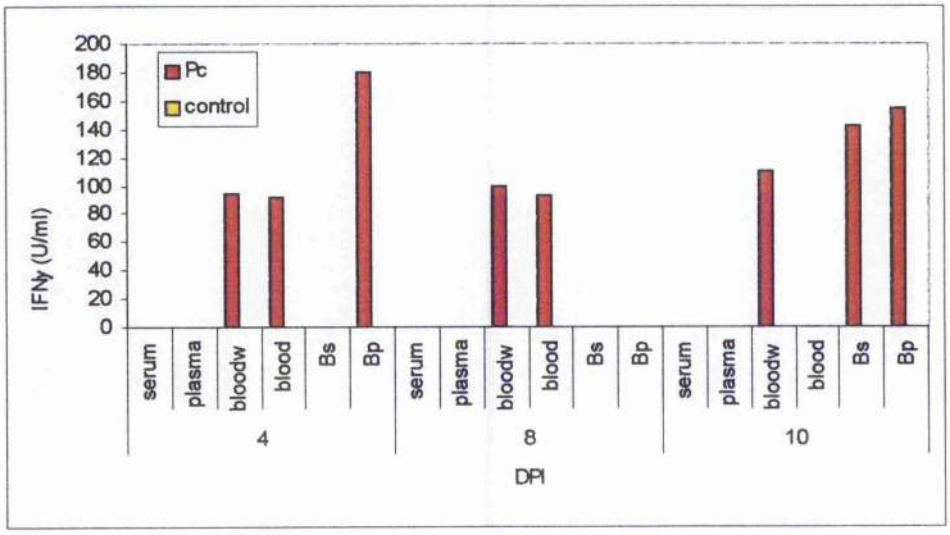


Figure 7.11. Circulating TNFα levels in different specimens from *P. c* AS-infected female ICR mice. Animals were infected with $1x10^5$ pRBC. Results represent means of three wells containing pooled samples of two mice per time point. Bs; packed cells remaining following the removal of serum, Bp; packed blood cells remaining following the removal of plasma, Bw; whole blood with ddH_2O (100µl/ml), blood; whole blood without ddH_2O. Results shown were obtained following thawing the samples for the sixth time.



a



b

Figure 7.12. a and b. Circulating IFNγ levels in *P. c* AS-infected female ICR mice. Bloodw; whole blood with ddH_2O, blood; whole blood without ddH_2O, Bs; packed blood cells remaining following removal of serum, Bs; packed red cells remaining following removal of serum. Figure 7.12.a; results obtained following second thawing of the samples, figure 7.12.b; results obtained following sixth time thawing of the samples. Results represent of three wells containing pooled samples of two mice at each time point.

Discussion:

As mentioned previously, one of the aims of this research was to investigate the hypothesis that NISV suppressed TNFα production by stimulated macrophages, suggested by the manufacturers of NISV (Dr. J. Brewer, Division of Immunology, Infection & Inflammation, Western Infirmary, Glasgow). It was show in Chapter 4 that NISV did indeed suppress circulatory TNFα production in *P. c* AS-infected male BALB/c mice. The studies done here aimed at confirming NISV-induced suppression of TNFα production *in vitro*.

NISV effects on splenocyte proliferation *in vitro* revealed several properties of the NISV, among these is the fact that NISV do not mix easily with the culture medium. This could be due to their hydrophobic outer layer (Brewer, 1993) (see Materials and Methods) forming a surface interphase. In fact, when adding NISV to culture medium in Bijoux tubes, a distinguished white fluid would be seen which did not mix readily with the culture medium unless well shaken. The difficulty of NISV to mix with the culture medium could explain why, upon adding small volumes of NISV to the wells, (following addition of cells and ConA) a dose-dependent suppression of splenocyte proliferation resulted. On the other hand, mixing NISV vigorously with the culture medium in relatively large volumes resulted in total suppression of splenocytes proliferation regardless of the NISV concentration indicating that addition of small volumes to the wells as a last step resulted in NISV floating on the surface of the culture medium and hence producing only a local suppressive effect on the underlying splenocytes. This was also suggested by the difference in splenocyte proliferation results obtained from protocols 3 and 4. Adding NISV to the splenocytes after the addition of ConA resulted in some proliferative response while adding NISV to splenocytes before the addition of ConA led to total suppression of proliferation although NISV were added in relatively large volumes in both cases (50µl) suggesting that the NISV formed a barrier between the cells and ConA in the latter case.

Another property of NISV revealed by these studies was that the NISV-induced suppression of splenocytes proliferation was not due to NISV toxicity but rather other mechanisms were involved. An important point noted is that the splenocyte suppression was dependent on the exposure time to NISV.

In the *ex-vivo* work, however, NISV were seen to suppress splenocyte proliferation of the treated group only slightly and at certain time points only. This slight statistically non-significant suppression could be a genuine finding or it could merely represent a normal biological variation. Biological variation was indeed obvious in the response of the two control groups PBS and NISV on day 1 post infection when their proliferation was much lower than the rest of time points. It could also, however, represent errors made by the operator in counting the splenocytes.

Among agents known to suppress splenocyte proliferation are PGE2 and NO (Riley *et al*, 1989, Ahvazi *et al*., 1995). It was seen that the NISV did indeed induce NO production from splenocytes, bone marrow-derived macrophages and liver adherent cells and that NO induction by NISV was dose-dependent. With the use of LNMMA *in vitro*, it was seen that some recovery of splenocyte proliferation was achieved. This was true for cells exposed to the lowest concentration of NISV (2.5µl/well), while no proliferation was seen in presence of a higher concentration of NISV. This could be due to the fact that LNMMA dose (500µM/well) used was not enough to block all NO production induced by NISV concentrations of 5 or 10µl/well (25 or 50 µl/ml respectively). This work could, therefore, be modified by exposing the splenocytes to NISV for various time intervals and to several concentrations of LNMMA with subsequent measurement of splenocyte proliferation and NO levels in the supernatants. The mechanism involved in the induction of NO by NISV is not known and more work is needed to investigate this. It, however, seems that NISV stimulate production of NO from splenocytes by direct contact. This hypothesis could explain why a dose-dependent proliferation response was seen upon addition of NISV in small volumes to the splenocytes and why the splenocyte proliferative response was also dependent on the exposure time to NISV. In other words, it seems that addition of the NISV to the splenocytes leads to accumulation of the NISV on the surface of the culture medium and through close contact with the splenocytes underneath, they

induce the production of NO form these splenocytes which in turn leads suppression of splenocytes proliferation. It, therefore follows that the amount of NO induced is dependent on the number of cells stimulated by NISV. Mixing the NISV well with the splenocytes allowed a greater number of splenocytes to be stimulated with the NISV with production of sufficient amounts of NO to cause total suppression of the splenocytes. This hypothesis could be investigated by adding NISV to splenocytes according to the protocols mentioned earlier and measuring the NO levels in the supernatants of the splenocytes. The results also indicate that 1 hr incubation with NISV does not induce the production of sufficient amounts of NO to cause splenocyte suppression. Three hour or longer incubation, on the other hand, seems to be sufficient to stimulate the signal for NO production and hence causing splenocyte suppression even after the removal of NISV. It would, therefore be useful to expose the splenocytes to various concentration of NISV for various lengths of time and measuring NO levels in the supernatants along with the splenocyte proliferation. It would also be useful to measure the levels of PGE2 to if splenocyte suppression is mediated partly by it.

Another important finding here is that, contrary to the previous findings of NISV causing TNFα suppression (Roberts, Brewer and Alexander, 1995 patent application), no such effect was seen in these experiments. Among cells tested in previous studies were murine bone marrow macrophages from female BALB/c mice and human peripheral blood lymphocytes (Roberts, Brewer and Alexander, 1995 Patent). Murine bone marrow derived macrophages from female ICR mice were tested here but no TNFα was detected not even in response to LPS (3μg/ml) or LPS (250ng/ml) and IFNγ (100U/ml) although the same TNFα antibodies were used. In some of these experiments it was seen that NISV induced the production of TNFα. Also previous studies have only reported low level detection of TNFα in the range of 0.09-0.12 U/ml and 0.5-3ng/ml while the levels detected here were greater in the range of 4-18U/ml. Previous studies (Roberts, Brewer and Alexander, 1995, patent application) also showed detection of TNFα at several time points (1.5, 4, 20, 24, 40, 48 and 60hr) which was never achievable in the experiments done here. The reason could be due to the use of a different strain of mice which means the above work should be repeated using BALB/c mice. The difficulty faced in measuring TNFα levels in the

studies presented here could also be attributed to the rapid clearance half life of the circulating TNFα (Beutler *et al.*, 1985) which could also explain the detection of TNFα in BM-derived macrophages at two hrs but not at 4 and 6 hr (Figures 7.9 a and b). It must be mentioned, however, induction of TNFα production by NISV agrees with the finding of their induction of NO production since NO has been shown to be a downstream product of TNFα (Rockett *et al.*, 1992).

In view of the above results, it could be concluded that NISV induced the production of NO form splenocytes *in vitro* which could explain at least partly the splenocyte proliferation suppression by NISV. No such conclusion, however, can be made with regards to NISV effects on splenocyte proliferation and their NO production in *ex-vivo* settings. The low proliferation response of the Pc and PcV groups observed on days 10-17 agrees with the previous reports on malaria-induced immunosuppression (Côrrea *et al.*, 1980).

It can also be concluded that NISV do not cause TNFα suppression from macrophages obtained from female ICR mice and that TNFα detection in general is in fact difficult. This work needs to be done on female BALB/c mice to confirm the previous results (Roberts, Brewer and Alexander, 1995, patent application)

The results obtained by measuring cytokines in different types of blood samples (whole blood, serum and plasma) and after repeated freezing and thawing of the samples clearly indicate the need to explore this area in order to interpret the ELISA results accurately. TNFα levels were detected in whole blood especially with addition of ddH2O and also in the packed red cells remaining after removal of plasma and serum, while no TNFα was detected in the serum or plasma samples. This could be due to the release and detection of the membrane bound TNFα (Decker *et al.*, 1987, Kreigler *et al.*, 1988) upon destruction of the blood cells (peripheral blood mononuclear cells). It could also due to induction of TNFα production by damaged erythrocytes. Damaged erythrocytes had been shown to induce TNFα production by peripheral blood mononuclear cells (PBMC) with parasitized red cells inducting x 200 TNFα levels induced by non-parasitized red cells (Bate *et al.*, 1994). IFNγ was also detected in whole blood while it was absent in plasma and serum samples. Upon

binding with its receptors IFNγ is rapidly internalized and eventually degraded in the lysosomes (Anderson, 1983). The levels of IFNγ detected in whole blood and in liquidized packed cells remaining after removal of serum or plasma could reflect the release of internalized IFNγ upon damage of PBMN by freezing. Detection of IFNγ after sixth time thawing of the samples could therefore represent release of IFNγ from a greater number of damaged PBMN following repeated freezing and thawing. The absence of IFNγ in the Bp samples after sixth time thawing could be due to IFNγ levels being above range of detection. This could be clarified by using serial dilutions of this sample.

In view of the above results, it becomes clear that more work is required in comparing cytokine levels in different types of blood samples and after repeated freezing and thawing of these samples. This could help interpret the results obtained by different studies and to explain the disagreement seen between some studies with regards to circulating cytokine levels.

Chapter 8

General Discussion

The studies presented in this thesis cover two main areas: firstly, they investigate the effects of pre-infection treatment with empty NISV on the course of *P. chabaudi* AS malaria in male BALB/c mice and secondly, they examine a hypothesis suggesting that NISV suppress TNFα production from macrophages.

The use of empty NISV in these studies follows an accidental finding that pre-infection treatment with NISV in *Toxoplasma gondii* –infected mice resulted in a less degree of weight loss in the treated mice compared with the non-treated mice (Brewer, 1993). Further studies were planed to investigate the effects of NISV on other pathologies characterized by weight loss and high TNFα levels such as septic shock (Beutler and Cerami, 1989) (Roberts, Brewer and Alexander, 1995, patent application). Malaria, on the other hand, imposes a huge burden on people morbidity and mortality and there is a great difficulty to control the disease for several reasons among which are; antigenic variation exhibited by the parasites, parasite resistance to anti-malarial drugs and mosquito resistance to insecticides. It is, therefore, important to improve our current methods of combating the disease and also to explore new avenues. Investigating the effects of NISV was considered as one of these new methods since malaria is also characterized by weight loss and high TNFα levels (Grau *et al.*, 1987, Grau *et al.*, 1989, Kern *et al.*, 1989, kwiatkowski *et al.*, 1990).

The hypothesis that NISV suppressed TNFα production was based on previous reports associating high TNFα levels with cachexia (Buetler *et al.*, 1985, Feinstein *et al.*, 1993). In fact high TNFα levels have been associated with several pathologies including septic shock (Beutler and Cerami, 1989), HIV, (Lahdevitra *et al.*, 1988), end stage chronic heart failure (Levine *et al.*, 1990) and parasitic infections (Scuderi *et al.*, 1986) including malaria.

The concept of suppressing TNFα in malaria is not new and anti-TNFα monoclonal antibodies have been used experimentally in both human and murine malaria (Grau *et a.*, 1989). It was thought that if NISV did indeed suppress TNFα levels, then it would provide several advantages over the anti-TNFα monoclonal antibodies including the

cheapness and the ease of their synthesis and storage. Also, anti-TNFα antibodies in some studies on human cerebral malaria increased the neurological sequelae which was thought to be due to prolongation of TNFα in circulation (van Hensbroek *et al.*, 1996). By suppressing TNFα production all together, NISV should not risk such serious consequences.

The initial studies conducted at the first venue (Strathclyde University) showed that pre-infection treatment of *P. c.* AS-infected mice resulted in a less severe course of illness as measured by a lower degree of weight loss, a lower level of parasitaemia and a higher health status score. The rate of mortality, however, was not significantly different between the two groups. These studies also showed a lower level of circulating TNFα in the NISV-treated mice agreeing, therefore, with the previous reports associating high circulating TNFα levels with the severity of malaria pathology (Grau *et al.*, 1987, Grau *et al.*, 1989, Al-Yaman *et al.*, 1990, Kern *et al.*, 1989,). The results also agreed with the hypothesis that NISV suppressed circulating TNFα levels (Brewer, personal communication). In repeat studies which were conducted at Glasgow University, however, although there was a reduced circulating level of TNFα in the NISV-treated group, the disease severity was greater in the treated group with regards to the same parameters of weight loss, level of parasitaemia and health status score. As mentioned previously in Chapter 3 a number of possibilities were considered to explain the difference in the clinical outcome in the experiments. Among these were the possibility of infection of the animals at the first venue by unknown pathogens, variable PABA levels in the water supply and errors in the synthesis of NISV. All these possibilities however, were eliminated but the difference in the clinical outcome in different experiments persisted. Another factor considered as a possible cause for the difference in the clinical outcome was the size of vesicles. The size of NISV had been reported to determine which arm of the T helper cell immune response was enhanced (Brewer *et al.*, 1998). Vesicles of >225nm in diameter preferentially induced Th1-type responses, while vesicles of <155 nm diameter induced Th2-type response. This possible explanation, however, was rejected for several reasons. The relationship between the NISV size and the arm of T helper cell response promoted has been observed with NISV containing

antigen (Brewer *et al.*, 1998), while in the experiments presented here, empty NISV were used. Also, the empty NISV used here were of mixed sizes, which had been observed previously to suppress TNFα levels (Brewer, 1993) and not to enhance either arm of the Th cell immune response. Furthermore, even in the experiments where the NISV-treated group developed a worse disease than the untreated group, TNFα was in fact suppressed.

Another possibility was that NISV caused suppression of TNFα in an uncontrolled manner. As mentioned previously, in murine malaria, TNFα has been reported to play a dual protective and pathological role depending on its levels (Stevenson and Ghadirian, 1989), timing and site of production (Jacobs *et al.*, 1996). The dual action of TNFα could be explained on the basis of the known functions of TNFα. TNFα has been reported to cause the production of NO (Rockett *et al.*, 1991, 1992). NO has been shown to cause asexual blood stages killing *in vitro* (Rockett *et al.*, 1991) and resistance against asexual blood stage malaria in mice has been reported to correlate with NOS mRNA expression in the liver (Jacobs, Radzioch and Stevenson, 1995). High levels of circulating nitrates, however, have been reported in association with the depth and duration of coma in human malaria (Al-Yaman *et al.*, 1990) due to NO interference with brain neurotransmitters (Clark *et al.*, 1991). NO also cause vasodilatation (reviewed by Moncada, Palmer and Higgs, 1991) which could be thought of as a beneficial action by counteracting the mechanical obstruction caused by binding of parasitized erythrocytes to endothelium. One can envisage, however, that excessive vasodilatation can lead to hypotension and shock as seen in septic shock (Beutler and Cerami, 1988). In addition, TNFα has been reported to be an endogenous pyrogen (Dinarello *et al.*, 1986) and indeed anti-TNFα antibodies in human malaria have been reported to prevent fever in children with cerebral malaria (Kwiatkowski *et al.*, 1993). Febrile temperatures have been shown to suppress the growth of *P. falciparum in vitro* (Kwiatkowski, 1989) but it can also induce epileptic seizures. TNFα has also been reported to suppress erythropoiesis and promote erythrophagocytosis in murine malaria (Clark and Chaudhri, 1988; Miller *et al.*, 1989). Although the resultant reduction in red blood cells could be thought of a mechanism to reduce parasitaemia and hence control the infection, it can also be seen that it can lead to severe anaemia (Taverne *et al.*, 1994).

Add to the above the fact that in *P. c.* AS infection, protection is associated with sequential appearance of Th1 and Th2 responses (Langhorne *et al.*, 1989). Th1 cells produce IL-2 and IFNγ (Mosmann *et al.*, 1986) and IFNγ in turn enhances the production of TNFα from macrophages (Grau *et al.*, 1989, Murray *et al.*, 1985) and increases the expression of TNFα receptors (Tsujimoto *et al.*, 1986). This means that IFNγ and TNFα are important in the early stages of infection to reduce primary parasitaemia to sub-patent levels. Suppressing TNFα early in the infection, therefore, could result in harmful effects.

It follows that certain levels of TNFα are required at certain period during the illness to induce the changes needed to combat the disease but above a certain level (Clark *et al.*, 1987), TNFα induces the changes which are pathological to the host (Stevenson and Ghadirian, 1989). TNFα is, however, not the only molecule that has dual effects. Everything in nature studied so far shows dual effects depending on its levels including essential elements such as water and oxygen. Perhaps the problem with TNFα is that the range of its beneficial levels is very narrow. This means that if NISV do cause an uncontrolled suppression of circulating TNFα levels, then they could indeed be dangerous in some individuals due to excessive suppression of TNFα. NISV could also be dangerous in certain pathologies requiring the presence of Th1 response such as *Leishmania major* (Bretscher *et al.*, 1992). It also means that NISV should not be administered prior to infection but should be used therapeutically and only in those individuals exhibiting a severe disease. The amounts given should be titrated in the individual patients to prevent deprivation of the host of beneficial levels of TNFα needed to combat the disease. Therapeutic use of NISV could be beneficial not only in malaria but in any pathology associated with high circulating levels of TNFα such as septic shock (Beutler and Cerami, 1989).

A third possibility to explain the development of a more severe disease in the NISV-treated group despite a lower circulating TNFα levels could be that the disease severity in the two groups was not a genuine one but merely a chance event. Two points are in favour of this possibility. First the fact that when the experiments were combined, no real

difference was seen between the NISV-treated and untreated groups with regards to weight loss, level of parasitaemia, health status score and mortality and secondly the fact that differences were seen among animals of the same group. This means that in the system used here, NISV-induced suppression of TNFα did not produce a significant impact on the clinical outcome. It would, therefore, be interesting to examine NISV effects in systems such as ECM in CBA/Ca mice infected with *P. berghei* ANKA where suppression of TNFα by antibodies reduced the development of CM (Grau *et al.*, 1987c) outcome not only in malaria but perhaps in other pathologies such as septic shock (Beutler and Cerami, 1989).

To study the mechanisms of NISV-induced suppression of circulating TNFα, cytokines known to regulate TNFα levels such as IFNγ and IL-4 were also measured. IFNγ is produced by $CD4^+$ Th1 cells (Mosmann *et al.*, 1986) and is known to stimulate TNFα production by macrophages (Grau *et al.*, 1989, Murray *et al.*, 1985). If TNFα suppression were through NISV acting on Th1 lymphocytes, one would expect IFNγ levels to be low as well. However, IFNγ levels were found to be unaffected by NISV treatment. IL-4, on the other hand, is produced by Th2 cells (Mosmann *et al.*, 1986) cells. IL-4 has been described as an anti-inflammatory cytokine because of its ability to suppress pro-inflammatory cytokines. IL-4 was shown to suppress TNFα production from human PBMN (Hart *et al.*, 1993). If NISV induced TNFα suppression by acting on the Th2 cells, one would have expected circulatory IL-4 levels to be elevated in the NISV-treated group. Plasma IL-4 levels were found to be higher in the NISV-treated group, but they were high from day 13 on wards, while TNFα suppression was seen early in the infection around time of primary parasitaemia. Also, the results were obtained from one experiment only and further work would be needed before reaching a conclusion regarding the *in vivo* effects of NISV on circulating IL-4 levels.

It was hoped that the *in vitro* studies would shed some light on the mechanisms of the NISV-induced suppression of TNFα. Previous studies had reported suppression of TNFα production from murine peritoneal macrophages and human peripheral cells

(Roberts, Alexander and Brewer, 1995, patent application). TNFα suppression seen in these studies was reported at multiple time points including 1.5, 4, 24, and 48hr. In contrast, the *in vitro* studies presented here showed that TNFα was induced by NISV treatment of splenocytes, hepatic adherent cells and bone marrow-derived macrophages. The NISV induction of TNFα production agrees with the findings that NISV also induced NO production since NO is known to be a downstream product of TNFα (Rockett *et al.*, 1991). Induction of NO production by NISV explains the suppressive effects of NISV on splenocyte proliferation since NO has been reported to suppress lymphocyte proliferation (Ahvazi *et al.*, 1995). The studies showed that close contact was needed between NISV and splenocytes to cause suppression of splenocyte proliferation. It is not known whether external contact between NSV and splenocytes is sufficient or that NISV are actually engulfed by the macrophages where they stimulate intracellular mechanisms for NO production. Direct effect of NISV on the macrophages could explain why neither IFNγ nor IL-4 levels were affected by NISV treatment *in vivo*.

The metabolic fate of NISV is not known. This makes it difficult to interpret the above *in vivo* and *in vitro* findings. Combining the *in vitro* and *in vivo* findings together, it can envisaged that macrophages are stimulated by NISV to produce NO immediately (NO detected in the first 2hr *in vitro*). Later on it seems, however, that macrophages start to degrade the NISV. During this time macrophages seem to be some how paralyzed and unable to produce TNFα when stimulated with antigens such as malaria antigens. To test this hypothesis several modifications have to be introduced in this work. The *in vivo* and *in vitro* studies must be made very similar to each other unlike the studies presented here where *in vivo* administration of the NISV was long time (4 and 2 weeks) before infection, while in the *in vitro* studies NISV were added at the same time as the parasitized red cells. It would therefore, be useful and interesting to administer NISV *in vivo* on the same day of infection and measure both circulating TNFα and nitrate levels from day 0. Similarly, it would be interesting to expose splenocytes to NISV for several days *in vitro* before stimulation with ConA or parasitized red cells and measure NO and TNFα in the supernatants. It would also be very useful to label the NISV perhaps radioactively and trace their metabolic fate both *in vivo* and *in vitro* and elucidate when their degradation

by macrophages start. Since whole splenocytes were used in the *in vitro* studies presented here, it would also be useful to measure the levels of IFNγ and IL-4 in the supernatants.

The studies done on comparing the cytokine levels measured in different types of blood samples (plasma, serum, lysed whole blood and lysed packed blood cells remaining following removal of serum or plasma) and following repeated freezing and thawing of the samples alert toward standardization of methods of measuring the cytokines by ELISA among all operators (variation in methodology was noted, personal experience). The studies clearly show an effect for blood haemolysis on the levels of TNFα detected.

In Summary, these studies have shown a TNFα suppressive effect by the NISV *in vivo* but an inducing one *in vitro* which agrees with their NO production induction *in vitro*. To explain this difference between the *in vivo* and *in vitro* settings, further modified work as described above is needed. Further work is also needed to investigate the *in vivo* fate of the NISV. In addition, it would be useful to measure the levels of TNFβ which shares TNFα properties and receptors (Ferrante *et al.*, 1990, Rockett *et al.*, 1992) and the levels of membrane bound from of the TNFα (Decker *et al.*, 1987; Kreigler *et al.*, 1988). It would also be useful to investigate the NISV effects on models where TNFα suppression by anti-TNFα antibodies resulted in a beneficial outcome such as *P. bergei* ANKA infection in CBA/Ca mice (Grau *et al.*, 1987c). It would also be useful to investigate therapeutic effects of NISV. Finally, further work in needed to define the effects of repeated freezing and thawing of the samples and different types of samples on the cytokine levels detected.

Appendix 1:

Phosphate buffered saline (pH 7.2)

60g $Na_2HPO_4.12H_2O$

13.6g $Na_2HPO_4.2H_2O$

8.5g NaCl

Made up to 1 litre with de-ionised distilled water.

RPMI 1640 stock medium

10.39g RPMI 1640 powdered (with L-glutamine) (Gibco)

5.94g N2-hydroxyethylpiperazine-N-2 ethane sulphonic acid (HEPES) 25mM

Made up to 1 litre with de-ionised and distilled water, filter sterilised (Milipore/Gelman filter 0.22µm size) and adjusted to pH7.2

Incomplete RPMI 1640 medium

To stock RPMI 1640 medium the following were added:

11ml L-glutamine (Gibco)

5.5ml $NaHCO_3$

0.55ml 2-Mercaptoethanol (0.1M)

22ml Fungizone (Gibco)

2.2ml Gentamycin (Sigma)

In some of the RPMI 1640 stock instead of fungizone and gentamycin, penicillin and streptomycin were added.

Complete RPMI 1640 medium

Heat inactivated foetal calf serum (Gibco) was added to incomplete RPMI 1640 medium at a final concentration of 10%.

Giemsa's Buffer:

3g Na_2HPO_4

0.6g KH_2PO_4

Made up to 1 litre with distilled water and adjusted to pH 7.4

Giemsa's stain:

Giemsa's stain (Gurr BDHLtd) was diluted 1:10 in Giemsa's buffer.

PBS/Tween 20 (washing buffer):

Tween 20 (polyoxyethylene sorbitan monolaurate)

Made up to 1 litre with PBS to give a final concentration of 0.05%

Blocking buffer:

Heat inactivated foetal calf serum added to phosphate buffer saline to give a final dilution of 10%

Tris buffered saline

9g NaCl

1.6g Tris HCl

The pH was adjusted to pH 7.6 with HCl and made up to final volume of 1 litre with deionised and distilled water.

Carbonate/Bicarbonate coating buffer (0.05)

1.59g $Na2CO_3$

2.93g $NaHCO_3$

0.2g NaH_3

Made up to 1 litre with deionised and distilled water and adjusted to pH 9.6

Bicarbonate coating buffer

8.4g $NaHCO_3$

Made up to 1 litre with ddH_2O and pH adjusted to 8.2

NISV:

49.6mg monopalmityl-rac-glycerol

16.4mg dicetyl phosphate

46.6mg cholestrol

RPMI 1640 Malaria stock medium:

10.39g RPMI 1640 powdered medium (with L-glutamine) (Gibco)

5.94. N2-hydroxyethylpiperazine-N-2 ethane suphonic acid (Hepes) (25mM)

Made up to 960 ml with distilled water and filter sterilised.

Incomplete malaria medium:

To 100 ml aliquots of RPMI 1640 of malaria stock medium, the following were added:

4.2 ml 5% $NaHCO_3$

0.25 ml gentamycin (sigma)

complete malaria medium:

heat inactivated human AB serum was added to incomplete malaria medium at a final concentration of 10%.

Boyl's solution:

0.415g Ammonium chloride, made up to 5ml in ddH_2O (pH 7.2)

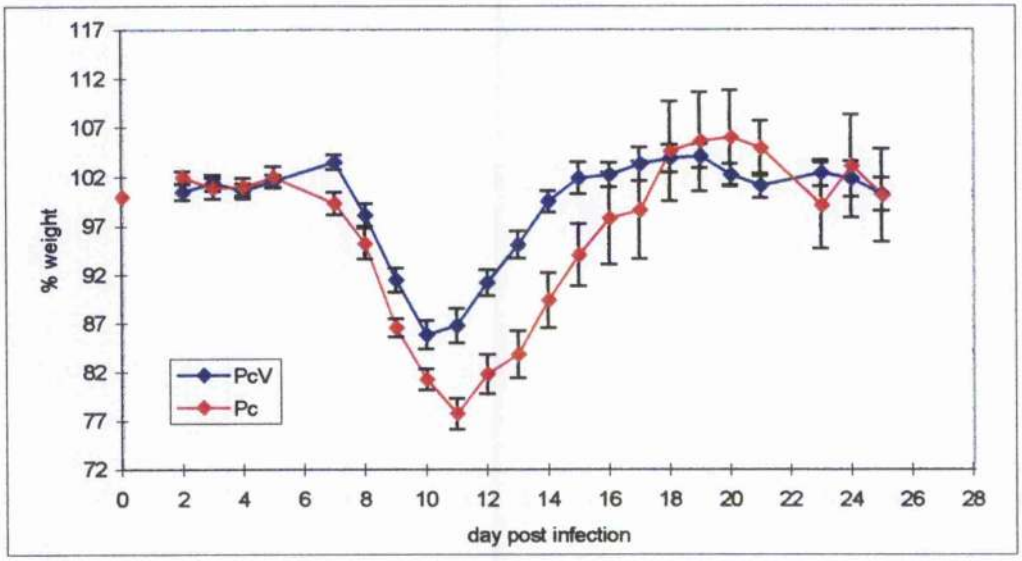
0.103g 2-amino-20(hydroxymethyl)-1,3-propendiol (Tris), made up to 50ml in ddH_2O (pH 7.2).

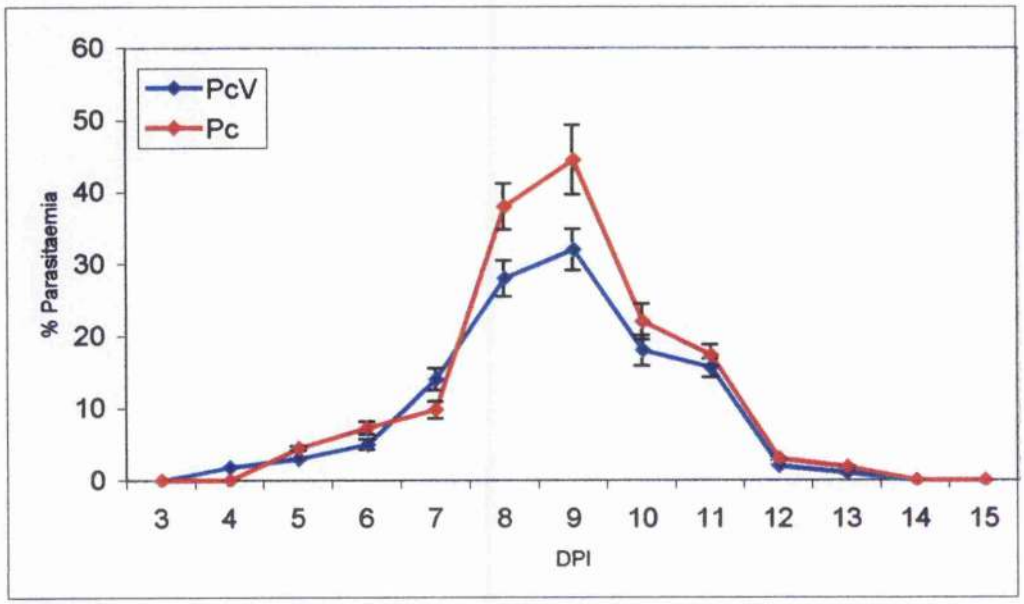
The two chemicals were mixed at a ratio of 9:1 and passed through a filter of 0.22µm before use

MTT:

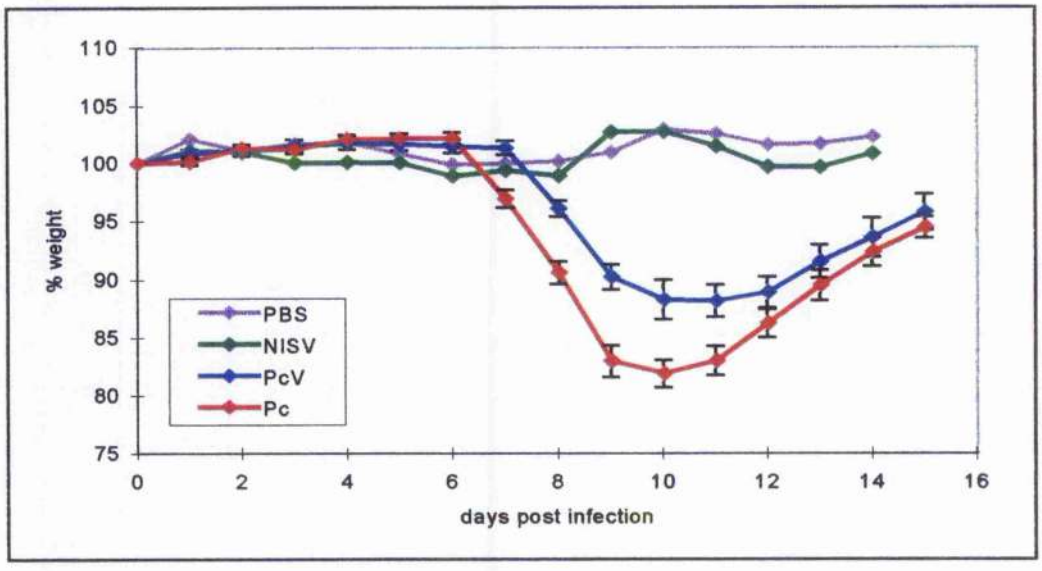
Made up to a concentration of 5mg/ml in ddH_2O. The solution was filtered immediately prior to use by passing it through a 0.2µm filter. The solution was finally diluted to 1mg/ml with RPMI 1640.

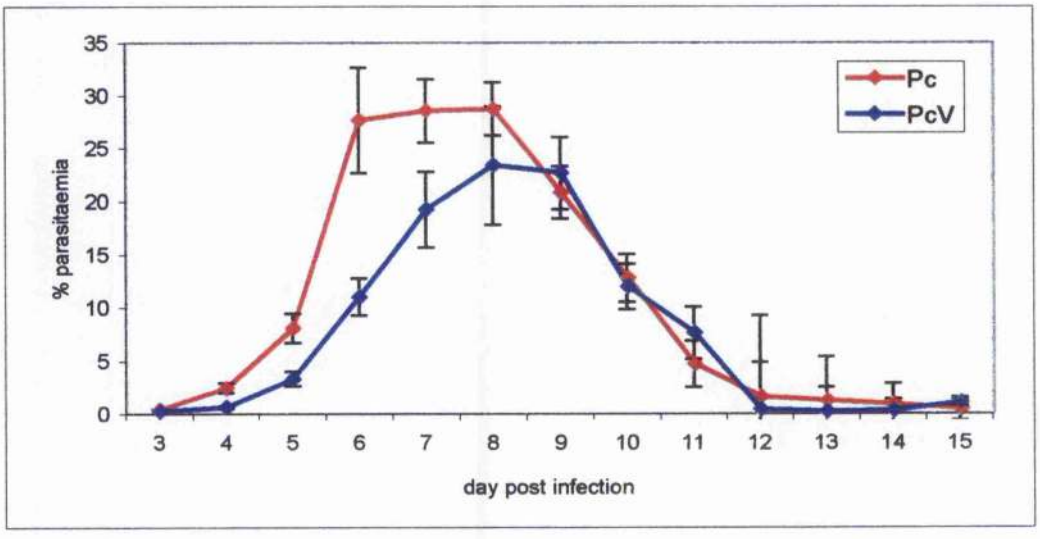
Appendix 2:



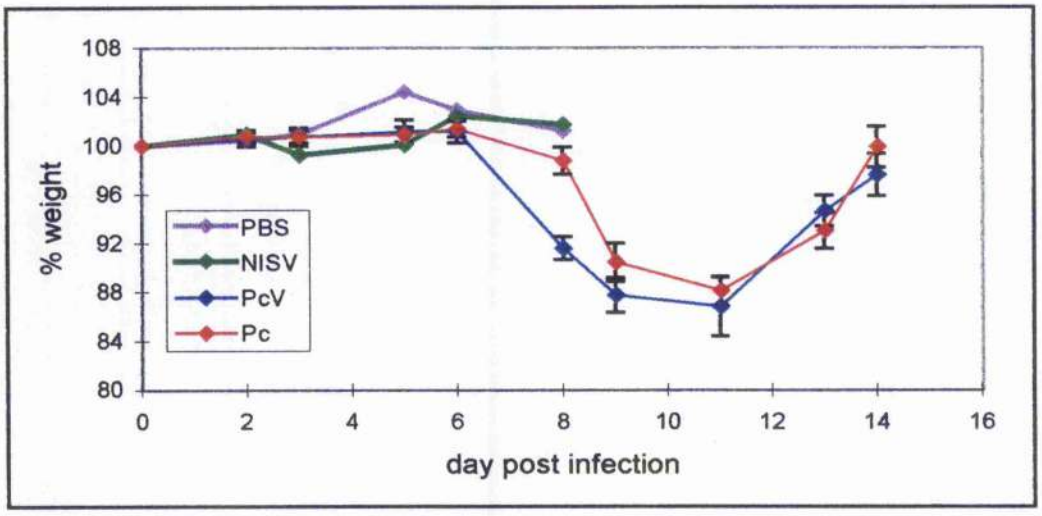


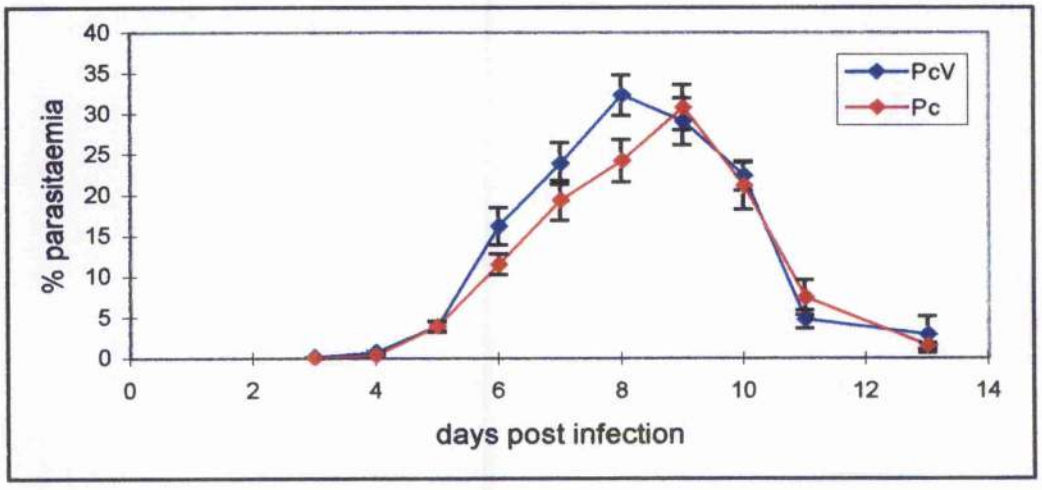
Experiment S.1: percent weight and parasitaemia in 10-12 week old male BALB/c mice infected with P. c. AS. Pc and PcV groups were infected with $1x10^5$ pRBC on day 0 and received 100ml of PBS or NISV respectively on days –28 and –14.



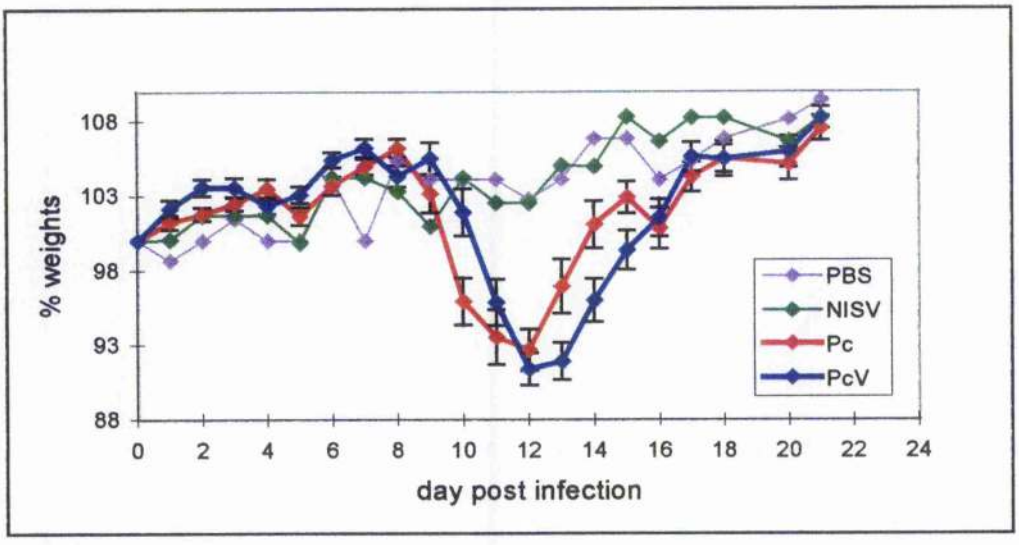


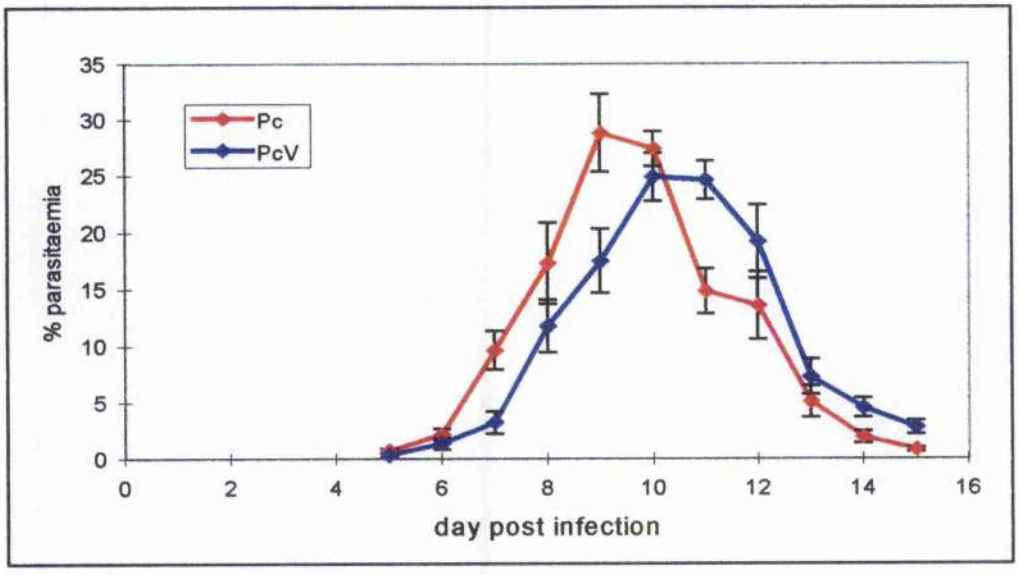
Experiment S.2: percent weight and parasitaemia in 10-12 week old male BALB/c mice infected with *P. c.* AS. Pc and PcV groups were infected with $1x10^5$ pRBC on day 0 and received 100µl of PBS or NISV respectively on days –28 and –14. PBS and NISV groups received 100µl of PBS or NISV respectively on days –28 and –14.





Experiment G.1: percent weight and parasitaemia in 10-12 week old male BALB/c mice infected with P.c. AS. Pc and PcV groups were infected with $1x10^5$ pRBC on day 0 and received 100µl of PBS and NISV respectively on days –28 and –14. PBS and NISV groups received 100µl of PBS and NISV respectively on days –28 and –14.





Experiment G.2: percent weight and parasitaemia in 10-12 week old male BALB/c mice infected with *P. c.* AS. Pc and PcV groups were infected with $1x10^5$ pRBC on day 0 and received 100µl of PBS and NISV respectively on days –28 and –14. PBS and NISV groups received 100µl of PBS and NISV respectively on days –28 and –14.

Appendix 3:

عَليلُ الجِسْمِ مُمْتَنِعُ القِيَامِ شَديدُ السُّكْرِ مِنْ غَيرِ المُدَامِ
وَزَائِرَتي كَأَنَّ بِهَا حَيَاءً فَلَيسَ تَزُورُ إلاَّ في الظَّلامِ[1]

Part of the poem by Al Mutanabbi (915-965 AD). Who wrote it in 959 AD describing fever he was suffering from during his stay in Egypt.

It translates as follows:

My visitor acts like she is shy for she does not visit me except at night.

Ref: Dar Sader. Beyronth.

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